IN VITRO STUDIES OF

FACTORS WHICH MAY INFLUENCE LIGAND BINDING,

FUNCTION, IMMUNOGENICITY

AND GENETIC REGULATION

OF THE BETA-2 ADRENERGIC RECEPTOR

IN ASTHMA

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This thesis is dedicated to my wife

Anne

and to my three daughters

Camilla Anne, Caronwen-Joy and Hayley

DECLARATION

I, PAUL CHARLES POTTER, hereby declare that the work on which this thesis is based is original (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

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30th May 1991

(DATE)

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ABSTRACT

This thesis records a series of experiments conducted to gain further insight into factors which influence the expression, ligand binding and functional activity of the beta-2 adrenergic receptor. These studies were prompted by previous reports that the postulated beta-2 adrenergic receptor abnormality in allergic asthma could be induced, induced by autoantibodies.

I established and optimised beta-2 adrenergic receptor ligand binding and functional assays in guinea pig lung membranes and then conducted an original study of beta adrenergic receptor expression in the guinea pig foetal lung. I found that beta adrenergic receptor expression in the foetal lung was dormant for 80% of the gestation period. After day 53 there was a surge in receptor expression which increased beyond term.

Using the guinea pig lung membrane as a source of beta-2 receptors, I investigated the inhibitory effects of serum from normal and allergic subjects on [^{125}I]cyanopindolol ([^{125}I]-CYP) binding to beta-2 adrenergic receptors and have found that ligand inhibition can be mediated by DEAE cellulose purified IgG fractions from both allergic and non-allergic individuals. Although a pilot study indicated that there may be significant differences between asthmatic and non-asthmatic sera, a larger prospective study showed no significant differences between the inhibition of [^{125}I]-CYP binding mediated by asthmatic and non-asthmatic sera ($p = 0.289$, Kruskal-Wallis test) and

there was a marked overlap in the values obtained. My data did not support an important role for autoantibodies to the human beta-2 adrenergic receptor in the pathophysiology of asthma.

In order to study genetic factors which may influence beta-2 adrenergic receptor expression regulation and ligand binding, I used a human beta-2 receptor cDNA probe (CLFV-108) to identify a 2.3kb beta-2 adrenergic receptor mRNA species in Wl-38 human diploid lung cells. Extending the genetic studies, I partially characterized 9 new mutant beta-2 receptors. These studies have confirmed that Aspartate 79 is important for agonist binding; that point mutation of Asn 6, increased antagonist binding, but point mutations of Asp 130, Ser 262, Asp 79 and Ser 346 had no effect on antagonist binding; and that Asn 6, Ser 262 and Ser 346 have normal isoproterenol stimulatable adenylate cyclase activity. Mutants involving Cys 191, His 172, Cys 190-191 and Asn 15 displayed impaired antagonist binding.

In order to explore conceptual models of the ligand binding domain and to investigate possible interactions of immunoglobulins with the beta-2 adrenergic receptor, I raised a mouse monoclonal antibody (designated B4A8-PWL) to a synthetic 20 amino acid peptide (spanning amino acid Aspartate 79-Methionine 99) which is located in the second hydrophobic transmembrane domain of the human beta-2 adrenergic receptor. Characterization of this IgM isotype monoclonal antibody revealed that whilst recognising the Asp79-Met99 peptide in an ELISA it did not recognize the intact beta-2 adrenergic receptor. It did, however, specifically recognised a 49kDa protein identified on Western blots of human and mouse cell lysates. I also transfected the human

beta-2 adrenergic receptor gene into B82 mouse L cells. These cells stably express human beta-2 adrenergic receptors at different levels. One clone stably expresses human beta-2 adrenergic receptors at higher levels than previously published (4000-6000 fmol/mg membranes) and the human beta-2 adrenergic receptors couple with mouse adenylate cyclase. I have designated this clone: Wt-7 Cape Town.

I also raised rabbit polyclonal antibodies to the Asp79-Met99 transmembrane peptide. These antibodies demonstrated [^{125}I]-CYP inhibition but, paradoxically, also stimulated adenylate cyclase activation in the Wt-7 Cape Town cells.

I used the Wt-7 Cape Town clone to investigate the functional effects of human allergic and non-allergic serum on beta-2 receptor coupled adenylate cyclase activation and have confirmed that certain sera exert markedly inhibitory effects. The inhibitory effects in this system, however, appear to be due to complement mediated cell damage via heterophile antibodies, rather than inhibition of beta-2 adrenergic receptor function. Purified IgG and products of activated platelets and lymphocytes were not inhibitory for adenylate cyclase activation but products of activated neutrophils were markedly inhibitory. I confirmed that IgG and sera from asthmatic and non-asthmatic individuals also inhibit [^{125}I]-CYP ligand binding to human beta-2 receptors using the Wt-7 Cape Town clone; that the degree of inhibition is related to the level of beta-2 adrenergic receptor expression in the Wt-7 Cape Town cells; and that the inhibition is non-competitive.

Finally, I examined the distribution of Ban 1 restriction fragment length polymorphism in 72 subjects in Cape Town and have found no significant difference between the gene frequency of either the 2.9 or 3.1 kb allele when allergic subjects were compared with non-allergic controls, but that the gene frequency for the two alleles was quite different to the gene frequency reported in North American subjects. Further studies, using a unique 326bp probe of the genomic sequence, suggested that the Ban 1 polymorphic locus was not only in the coding region of the gene but within a ligand binding domain.

These studies demonstrate that *in vitro* beta-2 receptor ligand binding, function and expression may be modified by factors which may operate at the level of gene regulation (e.g. steroids), the gene sequence (mutants) directly on the receptor (non-competitive inhibition by immunoglobulins) or via post-receptor (coupling) mechanisms or by factors which damage the cell membrane non-specifically. These factors may initiate or sustain the beta-2 adrenergic receptor dysfunction in human disease reported in previous studies.

ABBREVIATIONS

Acm	-	Acetamidomethyl
ABTS	-	2,2 Azinobis (3 ethyl benz thiazoline sulphuric acid) Diammonium salt
Bmax	-	Maximal ligand binding
cAMP	-	cyclic adenosine 3':5'-monophosphate
CLFV-108	-	Full length (1.2kb) coding region probe of human beta-2 receptor
CsCl	-	Cesium Chloride
ddH_2O	-	double distilled water
DEAE Cellulose	-	Diethylamino ethyl-cellulose
DMSO	-	dimethyl sulphoxide
dpm	-	disintegrations per minute
EGTA	-	Ethylene glycol-bis-(B-amino ethyl ether)N,N tetra-acetic acid
FCS	-	fetal calf serum
HbsAg	-	Hepatitis B surface antigen
HRPO	-	Horse radish peroxidase
HUCS	-	Human umbilical cord serum
IFA	-	Incomplete Freund's adjuvant
ITPG	-	isopropyl-B-D-thiogalactopyranoside
Kact	-	Concentration of agonist causing 50% of maximal stimulation
kb	-	kilobases
Kd	-	Dissociation constant
kDa	-	kilodaltons

KLH	-	Keyhole limpet haemacyanin
mRNA	-	messenger ribonucleic acid
NPGB	-	Nitrophenyl-p-guanidino benzoate
PAF	-	Platelet activating factor
PAGE	-	Polyacrylamide gel electrophoresis
PEG	-	Polyethylene glycol
PGE_1	-	Prostaglandin E1
PMSF	-	Phenyl methyl sulfonyl fluoride
RP-10	-	RPMI with 10% heat inactivated fetal calf serum
RPMI	-	Roswell Park Memorial Institute Culture Medium 1640
TBS	-	Tris buffered saline
TCA	-	Trichloroacetic acid
TE	-	Tris-EDTA buffer
TG1	-	E. coli strain
tRNA	-	transfer ribonucleic acid
Wt-7 Cape Town	-	B82 L cells expressing high levels (4000-6000fmol/mg) of human beta-2 adrenergic receptors
X gal	-	5 bromo-4 chloro-3 indolyl-B-D-galactoside
YT	-	Yeast Bactotryptone Sodium Chloride Medium
[^{125}I]-CYP	-	±[^{125}I] Iodo-cyanopindolol
[^{125}I]-HYP	-	[^{125}I] Iodohydroxybenzylpindolol

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Adrenergic innervation of the airways.

Catecholamines elicit their characteristic effects through the activation of effector cells via the adrenergic receptors.

Partial activation or failure of activation of effector cells could arise from impaired catecholamine release, poor delivery of circulating catecholamines to their receptors, or factors which compete with, or sterically hinder access of the catecholamine to its receptor. Structural abnormalities of the adrenergic receptor itself modifying its coupling with G proteins and adenylate cyclase, or post receptor defects may also result in an impaired effector response.

Before considering factors which block the adrenergic system, it is important to review the known mechanisms of adrenergic receptor activation. Control of the airways by the adrenergic system may be direct or via circulating catecholamines. The neurotransmitter of the direct system is noradrenaline, released by sympathetic nerves whose preganglionic nerve fibres arise from the upper six segments of the thoracic spinal cord, synapse in the middle and inferior cervical ganglia and the upper four thoracic paravertebral ganglia and whose post ganglionic nerves enter the lung at the hilum, intermingling with cholinergic nerves to form a plexus around airways and blood vessels

(Richardson 1979, Laitinen 1985, Barnes et al 1986c). In contrast to the dense innervation of the parasympathetic system, the sympathetic innervation is sparse. Histochemical studies show that human airways, unlike those of cats, have sparse adrenergic innervation of submucosal glands, bronchial arteries and smooth muscle (Partenen et al. 1982).

It is believed that adrenergic nerves probably influence bronchomotor tone indirectly, by an interaction with the cholinergic innervation at ganglionic or post-ganglionic level. Baker et al (1983) has shown that norepinephrine inhibits nicotinic neurotransmission in airway ganglia of the ferret trachea through presynaptic alpha receptors, and Vermeire and Van Houte (1979) has suggested that norepinephrine probably inhibits the release of acetylcholine from post-ganglionic nerve terminals. The fact that neither alpha- nor beta-adrenoceptor antagonists have any influence on airway smooth muscle tone in healthy individuals suggests that the role of sympathetic nervous control of airway smooth muscle is small under normal conditions. However, since a substantial number of asthmatics develop bronchoconstriction after administration of beta blockers, which may be reversed by atropine, it is suggested that in the asthmatic, unopposed sympathetic drive is critical to counteract parasympathetic effects (Grieco and Pierson 1971). These important differences between asthmatics and normal individuals in their requirements for unopposed beta adrenergic receptor stimulation and in the differences in the responses of their airways to beta receptor blockade, imply that factors which block the normal function of beta adrenergic receptor in human asthma are likely to be important and should be identified and explored.

The role of circulating catecholamines, particularly adrenaline, in maintenance of airway smooth muscle tone in asthma, has been stressed by the studies of Ind et al. (1985). Endogenous adrenaline is capable of effecting bronchodilatation, and infusion experiments demonstrate that adrenaline can antagonise the effects of inhaled histamine (Warren et al. 1984). Noradrenaline has no effect on airway tone in both normal and asthmatic subjects (Berkin et al 1985, 1986). Noradrenaline functions as a neurotransmitter and not as a circulating hormone. Adrenaline is secreted by the adrenal medulla and plasma adrenaline concentrations are normal at rest in asthmatics compared with age matched controls (Barnes et al 1982). It is also known that nocturnal wheezing correlates closely with the normal circadian fall in circulating adrenaline (Barnes et al 1980). Normal circulating concentrations of adrenaline probably protect against bronchoconstriction and endogenous adrenaline probably exerts its effect at the level of airway smooth muscle as well as on airway mast cells. In acute asthma (Ind et al. 1985), exercise induced asthma (Warren et al. 1982) and isocapnic hyperventillation (Barnes et al. 1981a) there is no significant rise in plasma catecholamines as would be expected in normal individuals, but a normal catecholamine response to hypoglycaemia and histamine infusion is observed. There would appear to be a selective or partial defect in adrenaline release in asthmatics which may be a contributory factor, but is not a primary cause of the asthma.

The distribution of beta adrenergic receptors in the lung increases progressively from the trachea to the terminal bronchioles (Barnes et

al. 1983) and relaxation of human central and peripheral airways is mediated only by beta-2 adrenergic receptors (Zaagsma et al. 1983, Goldie et al. 1984), which are autoradiographically the predominant airway receptors of the human airway smooth muscle, from bronchi to terminal bronchioles (Carstairs et al. 1985).

Although intact function of beta-2 adrenergic receptors on smooth muscle is generally considered to be of critical importance in asthma, the function of beta-2 adrenergic receptors on other cells in human airways must also be considered. Beta agonists stimulate ion transport on human epithelium (Knowles et al. 1984), inhibit antigen induced mast cell mediator release from passively sensitised human lung fragments (Butchers et al. 1980) and also from isolated lung mast cells (Peters et al. 1982). Beta adrenergic receptor integrity is also important for the inhibition of pulmonary oedema (Persson et al. 1982) and increased airway microvascular permeability induced by mediators of anaphylaxis.

1.2 Factors influencing beta adrenergic receptor number and functional integrity.

Since the hypothesis of Szentivanyi (1968) that asthma was a disease of "impaired beta adrenergic receptor function" or "partial receptor blockade", there have been many studies investigating beta-2 receptor numbers and function in human asthma and in guinea pig asthma models. The major findings of the reported studies of beta adrenergic

receptors in human asthma are listed sequentially in Table 1, and in guinea pig asthma models, in Table 2.

While many of the studies listed in Tables 1 and 2 indirectly support Szentivanyi's hypothesis by demonstrating reduced beta receptor number and/or function, the original suggestion that there was a primary defect in β receptor function in asthma has never been substantiated.

Svedmyr (1984) was unconvinced that beta receptor abnormalities previously reported are of any clinical significance and has questioned whether a beta-2 receptor 'abnormality' contributes to the pathophysiology of asthma at all.

Since reports of cholinergic and alpha adrenergic hyperresponsiveness (Henderson et al. 1979, Kaliner et al. 1982) in asthma, and the discovery of non-adrenergic non-cholinergic (NANC) airway innervation (Coburn and Tomita 1978, Taylor et al. 1984), the relative importance of different neural, cellular and humoral pathways in the regulation of airways tone is not clear. Consequently the effects of a defect in any one of these components cannot be viewed in isolation.

The importance of a sound understanding of human beta-2 receptor function in relation to drug therapy in asthma has recently been stressed by Wang et al. (1991) and Sears et al. (1991), who have drawn attention to dangers of regular beta-2 agonist treatment in asthma. They have found that regular inhalation of a beta

sympathomimetic agent is associated with a deterioration in the majority of asthma subjects, in a double blind, placebo controlled randomised cross-over study.

In order to monitor the effects of drug therapy on beta receptor responsiveness, there is a need to examine the validity and clinical relevance of current methods of assessment of beta-2 adrenergic receptor function in asthmatics, most of which are indirect, and to develop reliable, more direct methods. In order to understand the basis of beta-2 receptor dysfunction in the pathophysiology of asthma, the role of serum factors, products of inflammation and beta agonists on human beta-2 receptors requires critical appraisal.

In reviewing the literature it is not clear to what extent the reported beta adrenergic receptor hyporesponsiveness is primary (i.e. genetic) or secondary (i.e. acquired), whether it is total or partial (organ selective), or whether abnormal receptor numbers and functional responses found in vitro, are indeed of any clinical significance. Furthermore, a specific structural or molecular abnormality of the beta-2 adrenergic receptor has never been described in any human or animal disease.

In order to place my own studies in perspective with what is already known about the nature and regulation of beta-2 adrenergic receptors, it is important at the outset to review the factors known to regulate the beta-2 adrenergic receptors. It is convenient to consider the published biochemical factors in this introductory chapter and to

address the factors which are important in the genetic expression of the beta-2 receptor in Chapter 4, in which I have discussed genetic aspects of the human beta-2 receptor.

In 1948, Ahlquist classified the adrenergic receptors as alpha and beta based on the relative potencies of the adrenergic agonists norepinephrine, epinephrine and isoproterenol on effector cells. Typical alpha adrenergic responses which he observed were smooth muscle contraction in most tissues, for which epinephrine and norepinephrine were the most potent and isoproterenol the least potent. By contrast, the beta adrenergic receptors typically mediated smooth muscle relaxation for which isoprenaline was more potent than either epinephrine or norepinephrine.

In 1967 Lands et al. reported two distinct beta receptor subtypes. By comparing the effects of various sympathomimetics on laboratory animals such as the rabbit, dog, guinea pig and rat they found a positive correlation between the effects on adipose tissue and cardiac stimulation (Beta-1) and between the effects of bronchodilatation and vasopression (Beta-2) and concluded that the receptor in each pair was the same. It is now known that even within a single organ, e.g. the lung, beta receptors may exist in both subtypes, however it is not known as yet whether more than one receptor subtype can be expressed on the same cell. The human beta 3 adrenergic receptor has recently also been characterised by Emorine et al. (1989). Novel beta adrenergic receptor agonists having high thermogenic anti-obesity and

anti-diabetic activities in animal models are among the most potent stimulators of the beta-3 adrenergic receptor.

With the development of sensitive high affinity radioligand assays for measuring beta adrenergic receptor number (Levitsky et al 1974, Lefkowitz 1974, Auerbach et al 1974) and the development of functional assays to measure beta adrenergic receptor mediated adenylate cyclase activation (Salomon 1979), it soon became apparent that beta adrenergic receptors were present on almost all cells studied, and that their numerical expression and function was not only variable in different tissues studied, but subject to hormonal, pharmacological, and physiological processes. Furthermore, it was also found that under certain circumstances refractoriness, desensitization or tolerance to catecholamine stimulation occurred (Su et al. 1980).

Stiles et al. (1984) have reviewed the published factors influencing beta adrenergic expression and function under two main headings: Homologous regulation of receptor number and function by hormones and/or drugs that bind to receptors directly (e.g. epinephrine or propranalol) and heterologous regulation of the receptor by hormones or drugs that do not ordinarily bind to the receptor (e.g. glucocorticoids). Biochemical and cellular mechanisms of homologous regulation of beta receptors may involve uncoupling from the G-proteins, internalisation of the receptors (Chuang and Costa 1978), and sequestering of the receptor in vesicles away from the components of the adenylate cyclase system (Stadel et al 1983b). Fusion studies using the Xenopus laevis erythrocyte (Strulovici et al. 1983)

have investigated the functionality of sequestered receptors. Stadel et al. (1983) also demonstrated that agonist induced desensitization involved beta adrenergic receptor phosphorylation of the turkey beta adrenergic receptor and Benovic et al. (1986) and Bouvier et al. (1988) have reported that a specific beta adrenergic receptor kinase is involved.

It was thus apparent that while homologous regulation occurred at a receptor level, heterologous regulatory factors could possibly operate at a pre-receptor (e.g transcriptional or post-translational level) or post-receptor level (e.g.coupling) with G protein or non-specific membrane effects.

Studies of factors influencing beta adrenergic receptor number and function which have been conducted in animals, in cell culture and in humans, during the past ten years are too numerous to discuss in this introduction. I have, therefore, summarised and grouped the major published studies which form an important background to my own studies in Tables 1-4 and will focus any discussion on the studies which address the issues which must be considered in evaluating my own work. I have listed the studies which focussed on human asthma (Table 1) separately from studies on beta-2 receptors in non-asthmatic subjects (Table 4). Broadly, previous studies may be grouped as follows: -

Table 1 Studies of beta adrenergic receptors in human asthma

Table 2 Recent studies of beta adrenergic receptors in guinea pig asthma models.

Table 3 Factors influencing beta adrenergic receptor number/function : animal studies.

Table 4 Factors influencing human beta adrenergic receptor number/function in non-asthmatics

A number of clear and consistent general observations about beta-2 receptor regulation are evident from the animal studies listed. In the first instance, adrenergic receptors may be down-regulated by agonists (Stadel et al 1983, Pecquery et al 1984, Su et al 1989). There is also an independent regulation of beta-1 and beta-2 adrenergic receptors by hormones, which may be organ specific e.g. glucocorticoids increase beta-1 receptor number in rabbit lung (Roberts et al 1985) and beta-2 receptors in the guinea pig lung (Salonen 1985) but do not increase beta-1 receptors in the rabbit myocardium. At critical developmental times, susceptibility to steroid induction of receptors may be dramatic (Cheng et al 1980). Differential susceptibility of beta adrenergic receptor subtypes to hormonal induction also occurs with thyroid hormone treatment: for example, rat heart beta-1 receptors are increased by thyroid hormone but no increase was observed in lung beta-2 adrenergic receptors (Scarpace and Abrass 1981, Tse et al 1980). However, the normal developmental increase in rat fetal lung receptors are dependent on thyroxine (Whitsett et al 1980).

TABLE 1 STUDIES OF BETA RECEPTORS IN HUMAN ASTHMA

Factor studied	Organ or cell	Effect on beta receptor	Reference
Propranalol administration to asthmatic	Bronchial hyperreactivity	Marked reduction in airway calibre	Zaid & Beal 1966 MacDonald et al. 1967
Catecholamine stimulation	Hyperglycaemic response	Inversely proportional to the severity of the disease in children	Inoue 1967
	Hyperglycaemic response	Asthmatic subjects have reduced response compared with normals	Lockey et al. 1967 Middleton & Finke 1968
Twin studies	Peripheral blood leucocytes	Reduced basal and isoproterenol stimulated adenyl cyclase activity in asthmatic compared with non asthmatic twin	Falliers et al. 1971
Epinephrine	Urinary cAMP	Urinary cAMP increased in normals but not asthmatics	Bernstein et al. 1972
Response to catecholamines	Leukocytes	Reduced cAMP response compared with controls	Parker & Smith 1973
Asthmatics off beta agonists	Heart	Reduced heart rate, responses to catecholamines	Larsson 1977
Chronic agonist (Albuterol)	Bronchodilator response	Subsensitivity induced by chronic administration	Nelson et al. 1977
Inactive vs active asthma	Lymphocytes	Beta receptors reduced in patients with active asthma but not inactive asthma	Kariman & Lefkowitz 1977
Reversibility of down regulation	Neutrophils	Discontinuation of adrenergic agonists reversed diminution of receptors induced by agonist therapy	Galant et al. 1978
B-agonist treatment	Lymphocytes	Reduced number	Brookes et al. 1979
Correlation with disease severity	Lymphocytes	Reduction in beta receptor number correlated with disease severity	Brookes et al.1979
Systemic receptor sensitivity	Pulse pressure	Larger doses of catecholamines required to raise pulse pressure	Shelhamer et al.1980

Factor studied	Organ or cell	Effect on beta receptor	Reference
β-receptor autoantibodies	Human sera	Inhibition of ligand [^{125}]IHYP binding, precipitation of solubilised receptors	Venter et al.1980 Fraser et al. 1981
Asthmatics vs controls	Neutrophils	No differences in beta receptor number or adenylate cyclase responsiveness in untreated asthmatics compared with controls	Galant et al.1980
Allergy	Autonomic nervous system	Decreased beta receptor hyporesponsiveness with increased cholinergic and α adrenergic responsiveness	Kaliner et al.1982
Diurnal response	Airway calibre	Response to inhaled epinephrine greater at 04h00 than 16h00	Barnes et al.1982
Aspirin induced bronchospasm	Lymphocytes	No difference in receptor number or isoproterenol stimulation	Wolfe et al.1982
cAMP response	Plasma	cAMP response to exercise blunted	Warren et al.1982 Ind et al.1983
Chronic obstructive pulmonary disease	Granulocytes	Reduced inhibition of lysosomal enzyme release by isoproterenol	Busse et al.
Exercise	Lymphocytes	Reduced cAMP response	Candell et al. 1984
Age of child	Lymphocytes	Age dependent increase in receptor number	Reinhardt et al.1984
β receptor autoantibodies patients with asthma	Human sera	Greater inhibition of ligand binding in certain asthmatic children	Blecher et al. 1984
Terbutaline treatment	Neutrophils	Reduced beta receptor number	Galant & Britt 1984
Inhaled Salbutamol	Lymphocytes	Reduced number	Reinhardt et al.1984
Terbutaline treatment	Lymphocytes	Reduced number	Sano et al.1984

Factor studied	Organ or cell	Effect on beta receptor	Reference
Allergen (house dust mite)	Lymphocytes	Reduced number and adenylate cyclase responsiveness	Meurs et al.1984 1987, 1988.
Diurnal variations	Lymphocytes	Circadian variation in affinity and number of receptors	Titinci et al.1984
Bronchial asthma	Lymphocytes	Reduced number	Sano et al.1984
Atopy	Nasal tissue turbinates polyps	Reduced number Lower affinity (in polyps)	Kinkel et al.1989
Atopy	Lymphocytes	Blunted cAMP response	Cundell et al.1984
Epinephrine levels	Plasma	Plasma epinephrine response to hospitalization blunted in asthmatics but plasma norepinephrine response not blunted	Ind et al.1985
Nocturnal catecholamine excretion	Urine	Diminished in asthmatics	Potsma et al.1985
Exercise induced asthma	Lymphocytes	Reduced number. Reduced responses (cAMP and fall in diastolic BP) to isoproterenol	Martinssen et al.1985
Atopic asthma	Pulse pressure	Asthmatics require a bigger dose of isoproterenol to raise pulse pressure 22mmHg	Davis et al.1986
Bronchial responsiveness	Lymphocytes	No correlation between receptor numbers and bronchial responsiveness	Davis et al. 1986
Exercise induced asthma	Lymphocytes	Failure of increase in receptors in asthmatics	Reinhardt et al. 1987
Ketotifen	Lymphocytes	Restoration of the up regulation of beta receptors following exercise	Reinhardt et al. 1987
Autoantibodies	Human sera	Inhibition of ligand binding in asthmatic and non-asthmatic subjects	Potter & Dakers 1987

Factor studied	Organ or cell	Effect on beta receptor	Reference
Active asthma and rhinitis	Lymphocytes	Reduced receptor number	Gamboa et al. 1987
Lymphocyte subsets	B cells	Receptor density 2- to 3-fold higher than in T cells	Korholz et al. 1988
Ketotifen	Lymphocytes	Increased catecholamine responsiveness	Polson et al. 1988
Asthmatic children	Lymphocytes	Low numbers of receptors	Bittera et al. 1988
Prednisolone	Lymphocytes	Enhanced recovery of lymphocyte beta-2 adrenoceptor responsiveness to beta agonists	Brodde et al. 1988
Autoantibodies	Human serum	No difference between ligand inhibition between asthmatics and controls.	Kokuba et al. 1989
Bronchodilator responsiveness	Bronchi	Attenuated responsiveness to beta agonists (isoproterenol and Fenoterol) not due to reduced receptor density	Spina et al. 1989
Allergic children	Lymphocytes	Reduction of beta receptor number unrelated to IgE levels or sensitivity to histamine challenge	Bittere et al. 1988
Ketotifen treatment	Human lung	Prevention of tachyphylaxis to inhaled sympathomimetics	Pauwels et al. 1989
Long term ketotifen treatment	Lymphocytes	Increased cAMP responsiveness to isoproterenol in asthmatics	Polson et al. 1988
Postmortem studies	Bronchial strip preparations	4-5 fold reduction in relaxant properties to isoproterenol and Fenoterol attributed to reduced smooth muscle beta-2 receptor function	Goldie et al. 1987

TABLE 2

RECENT STUDIES OF BETA ADRENERGIC RECEPTORS IN GUINEA PIG ASTHMA MODELS

Factors studied	Organ or cell	Effect on beta receptor	Reference
Ketotifen Treatment	Guinea pig lung	Prevention of terbutline induced decrease in receptor density	Koshino et al. 1987
Smooth muscle	Guinea pig lung	Significant decrease in beta receptor sites in bronchi and bronchiolar smooth muscle and in bronchial and in alveolar epithelium	Mu and Bi 1989
Allergen challenge	Guinea pig lung membranes	Significant reduction in beta receptor number in ovalbumin challenged group	Motojima et al. 1989
Epithelium	Guinea pig airway epithelium	Removal of epithelium removes protective effect of broncho-dilators in vitro in guinea pig	Goldie et al. 1986
Autoradiographic visualization	Airway epithelium and vascular smooth muscle	Significant quantitative reduction in [3H]-DHA binding in alveolar and conducting airway epithelium and bronchiolal smooth muscle in oval-bumin asthma model.	Gatto et al. 1987
Phospholipase treatment	Lung membranes	Reduction in beta receptor number 25%. Reduction in adenylate cyclase activity 68%	Taki et al. 1986
Asthma	Lungs	20% reaction of beta receptor number in asthmatic animals (ovalbumin model)	Barnes et al. 1980
Ketotifen	Lung	Prevention of down regulation of beta receptors induced by terbutaline	Koshino et al. 1988

TABLE 3

FACTORS INFLUENCING BETA ADRENERGIC RECEPTOR NUMBER/FUNCTION: ANIMAL STUDIES

Factor studied	Organ or cell studied	Species	Effect on beta receptor	Reference
Agonist treatment	Erythrocyte	Frog	Internalization	Chuang and Costa 1979
	Astrocytoma cells		Uncoupling internalization and degradation	Su et al. 1980 Doss et al. 1981
	Erythrocyte	Frog	Transfer into light vesicles	Stadel et al. 1983
	Erythrocyte	Turkey	Phosphorylation	Stadel et al. 1983
	Myocardium	Rat	Desensitization	Tse et al. 1979
	Aortic smooth muscle	Rabbit	Desensitization	Little et al. 1984
	Adipocyte beta-1	Hamster	Impaired coupling to adenylate cyclase	Pecquery et al.1984
	Cardiac myocytes beta	Rat	Energy dependent cycling of receptor	Limas & Limas 1984
	Airway muscle beta-2	Guinea pig	Decreased developmental sensitivity to catecholamines	Duncan et al. 1982
Glucocorticoid treatment	Heart B1	Cat	Enhanced positive inotropic effects	Kaumann 1972
	Lung	Rat	Increased number	Mano et al. 1979
	Foetal Lungs	Rabbits	Increased number (Beta 1)	Cheng et al. 1980
	Astrocytoma cells	Human	Increased number	Foster & Harden 1980
	Lung beta-2	Guinea pig	Increased number	Salonen 1985
	Lung beta-1	Rabbit	Increased number	Roberts et al. 1985
	Lung	Rabbit	Increased alveolar receptors but not airway receptors	Barnes 1985
	Myocardium B1	Rabbit	No increase	Barnes 1985
Denervation	Brain B1	Rat	Increased number	Sporn et al. 1976
6-OHDA	Heart B1	Rat	Increased number	Kajiyama et al.1982
Guanethidine treatment	Heart B1	Rat	Increased number Increased cAMP accumulation	Glaubinger et al. 1978
Axotomy (6-OHbA)	Splenic lymphocytes	Mouse	Increased number	Miles et al. 1984
Propranalol withdrawal	Heart B1	Rat	No evidence of up regulation	Kennedy & Donnely 1982
Hypothyroidism	Fat B1	Rat	Decreased number	Giudicelli 1978
	Erythrocyte B1	Turkey	Decreased number decreased adenylate cyclase	Furukawa et al.1980
	Heart B1	Rat	Decreased adenylate cyclase	Brodde et al. 1980

Factor studied	Organ or cell studied	Species	Effect on beta receptor	Reference
Thyroid hormone	Adult lung B2	Rat	No change)	Scarpace & Abrass
	Heart B1	Rat	Increase)	1981
	Lymphocyte B2	Rat	No change)	
	Foetal lung B2	Rat	Normal increase is thyroxine dependent	Whitsett et al.1980
	Heart B1	Rat	Increased	Tse et al 1980 Williams et al.1977
	Foetal lung B2		Increased with potentiation of surfactant release	Das et al. 1985
Senescene	Myocardium B1	Rat	Decreased catecholamine responsiveness	Scarpace et al.1986
Ischaemia	Myocardium B1	Guinea pig	Increased number	Maisel et al. 1985
Chronic stress	Pineal	Rat	Decreased number	Yocca & Fredman 1984
GTP	Fetal myocardium B1	Rabbit	Increased affinity for agonists	Hatjies et al. 1985
Estrogen	Preterm fetal lung B2	Rabbit	Enhanced agonist binding	Moawad AH et al.1985
Lectin treatment Con A and PHA	Brain	Rat	Increased density	Tsuchihashi H et al. 1985
IgG from Chagas disease	Myocardium	Rat	Binds to beta receptors and increased contractility	Borda et al. 1985
Alloimmune IgG(CFI)	Isolated oviduct	Mouse	Spontaneous motility inhibited	Borda 1984
Inhibition of microtubules (cytochalasin)	Heart cells	Chick	Blunted desensitization of contractile response and recovery	Marsh et al. 1985
Micro-organisms eg haemophilus influenza	Tracheal strip	Guinea pig	Reduction in isoprenaline induced inhibition of mediator release	Scheurs & Nijkamp 1984
Androgen	Kidney	Mouse	Increased number	Petrovic et al. 1981
Progesterone (Estrous cycle)	Ovary	Rat	Increased number	Jordaan 1981
Glucocorticoids	Peritoneal mast cells	Rat	Increased responsiveness	Tolone et al. 1979
Goldblatt hypertensive model	Kidney	Rat	50% fall in myocardial beta receptors	Woodcock et al. 1979 1980
Hypoxia	Myocardium	Rats	Decreased number and blunted responses	Voelkel et al.1981
Experimental diabetes	Skeletal muscle membranes	Rats	Decreased number (50%) but no effect on function	Olson et al. 1981
Chronic ethanol	Brain	Rat	Decreased	Banerjee et al. 1978
Ethanol withdrawal	Brain	Mice	Rebound increase	Kuriyama 1981
Anti-depressant therapy tricyclics	Brain	Rat	Decreased receptor number and response to isoproterenol	Hertz et al. 1981

Factor studied	Organ or cell studied	Species	Effect on beta receptor	Reference
Fetal/ Gestational changes	Lung	Rabbit	Increase in beta-1 receptors day 26-29	Giannopoulis 1980
	Lung	Rat	Increase in beta-1 and beta-2 receptors from day 18 to day 28	Whitsett et al. 1981
	Heart	Mouse	Increase in number and sensitivity from day 13 to 21	Chen et al. 1979
Cell differentiation	Adipocytes 3T3-L1	-	Increases in receptor number. Function dependent upon changes in N protein	Lai et al. 1981
Cell maturation	Reticulocyte-erythrocyte	Rat	Loss of catecholamine sensitive adenylate cyclase activity	Limbird et al. 1980
Interleukin 1	Cardiac myocytes	Rat	Depressed catecholamine sensitive adenylate cyclase activity	Gulick et al. 1989
Testosterone	DDT_1-MF-2 cells vas deferens	Hamster	Increased receptor number	Norris et al. 1987
Human IgG	Heart	Rat	Non competitive inhibition of ^{3}H-DNA binding	Sterin Borda et al. 1988

TABLE 4

FACTORS INFLUENCING BETA ADRENERGIC RECEPTOR FUNCTION/NUMBER IN NON ASTHMATICS

Factor studied	Organ or cell	Effect on beta receptor	Reference
Agonist	Leukocyte B2	Decreased receptor number	Davies & Lefkowitz 1981
Partial agonist acebutolol	Mononuclear cells	Decreased number	Basso et al. 1985
Glucocorticoids	Leucocytes B2	Increased receptor density	Davies and Lefkowitz 1983
		on granulocytes and lymphocytes, increased coupling efficiency	
	Lung cells W1-38	Increased number	Fraser and Venter 1980
	Astrocytoma cells	Increased number	Foster and Harden 1980
Beta blocker atenolol	Mononuclear cells	Increased number	Piantarelle et al. 1984
Propranalol withdrawal	Heart B1	Hypersensitivity to isoproterenol	Boudoulas et al. 1977 Nattel et al. 1977
	Heart B1	No evidence for hypersensitive response to catecholamines	Lindenfeld et al 1980
Hyperthyroid	Lymphocyte B2	No change in number or adenylate cyclase activity	Williams et al 1979
		Increased no. of receptors	Ginsberg et al. 1981
Congestive heart failure	Myocardium	Decreased number of receptors (50%) Decreased isoproterenol induced contraction 54-75%	Bristow et al. 1982
Cirannual variation	Mononuclear	High numbers in April/May Low numbers August and December	Haen et al. 1988
Cystic fibrosis	Sweat glands	Reduced number	Sato 1984
	Neutrophils	Reduced number	Tawara K et al. 1984
Phaeochromocytoma	Heart	Reduced number	Tsujimoto et al. 1984
	Lung	No effect	
Colchicine treatment	Leucocytes	Enhanced cyclic AMP response	Rudolph et al 1977
Age	Heart	Reduced sensitivity to isoproterenol with age	Vestal et al. 1974
Interleukin 1	Human lung tumor cells (AS49)	Increased receptor number	Stern and Kunos 1988
Interleukin 1	A549 cells	Potentiation of Isoproterenol induced increased cAMP	Nakane et al. 1990
Congestive cardiac failure	Lymphocytes B2	Decreased density	Mancini et al. 1989
Ageing	Leucocyte beta receptor	No reduction in responsiveness	Davis and Byerd 1990
Cardiomyopathy autoantibodies	Rat heart Beta-2 receptors	Reduced cAMP Reduction in ^{3}H DNA	Limas et al. 1989
Transplantation autoantibodies	Rat heart	Reduction in ^{3}H DNA binding	Limas et al. 1989

The demonstration by Stern and Kunos (1988) of a synergistic effect between the regulation of pulmonary β-adrenergic receptors by glucocorticoids and interleukin-1 suggests that regulatory factors of beta receptors are unlikely to be functioning independently, *in vivo*.

There are no animal models in the literature of autoimmunity to the beta adrenergic receptor but the studies demonstrating functional effects of human immunoglobulins on beta receptors on rat myocardium (Borda et al. 1984a) and of alloimmune sera on isolated oviducts (Borda 1984b) raise intriguing questions as to the possibility of similar antibodies occurring naturally. Besides endogenous factors, a number of exogenous factors e.g. ethanol (Banerjee et al 1979, Kuriyama et al 1981), antidepressant therapy (Hertz et al 1981), hypoxia (Voelkel et al 1981) and stress (Yocca and Fredman 1984) may have marked effects on beta adrenergic receptors. Furthermore, a number of as yet unknown factors operate at critical periods in gestation to stimulate beta adrenergic receptor development in the heart (Chen et al 1979) and in the lung (Giannopoulis 1980, Whitsett et al 1981).

Factors influencing beta receptors in human studies tabulated in Table 4, are consistent with the tissue and subtype specificity of the factors influencing beta adrenergic receptors reported in the animal studies (Table 3) but introduce the concept of a possibly disordered expression or function of beta adrenergic receptors in human diseases e.g. congestive heart failure (Bristow et al 1982, Mancini et al 1989), cardiomyopathy (Limas et al 1989), cystic fibrosis (Sato and Sato 1984, Tawara et al 1984) and phaeochromocytoma (Tsujimoto et al 1984).

Beta adrenergic receptors may also be affected by drugs used for therapeutic purposes (Boudoulas et al 1977, Piantarelle et al 1984, Basso et al 1985) and the sensitivity of beta-2 receptors to agonists may be modified by lymphokines e.g. Interleukin-1 (Nakane et al 1990).

Liggett et al (1988), in a detailed study of the correlations between tissue adrenoceptor function and the receptor and adenylate cyclase characteristics of easily accessible circulating cells, has shown that in the case of mononuclear leucocytes (MNL) the adrenoceptor densities on circulating cells reflect those on extravascular tissues in a subtype specific fashion, but that physiological inter-individual variation of adrenergic receptor characteristics were not of sufficient magnitude to alter sensitivity to epinephrine *in vivo*. In fact, adrenergic receptor adenylate cyclase from circulating cells (MNL and platelets) was found to be unrelated to the hemodynamic and metabolic responses to epinephrine in normal humans *in vivo*.

One of the most important questions which arise upon finding disordered beta receptor number and function in a given disease is whether these abnormalities have led to the disease, or whether they have occurred as a result of a disease process.

It is in the field of asthma (Table 1) that a review of the factors influencing beta adrenergic receptors is of particular relevance to the studies I have embarked upon. Increased sensitivity of asthmatic subjects to beta adrenergic receptor blockade following the administration of propranalol to allergic subjects was first reported

by Zaid and Beal in 1966. The development of the "beta adrenergic theory of atopic abnormality in asthma" was then developed by Szentivanyi (1968).

Early studies, conducted before radiolabelled high affinity specific ligands for beta receptors became available, reported reduced beta-2 receptor mediated responses in asthmatics. These included a reduced hypoglycaemic response (Inoue 1967, Lockey et al 1967, Middleton 1968), reduced isoproterenol stimulated adenylate cyclase activity in leucocytes (Falliers et al 1971, Parker and Smith 1973), reduced urinary cyclic cAMP (Bernstein et al 1972) and reduced cardiovascular responses to beta adrenergic stimulation (Larsson et al 1977, Shelhamer et al 1980).

Reduction in beta-2 numbers on lymphocytes of active asthmatic patients was reported by Kariman and Lefkowitz in 1977. These initial findings raised a critical question as to whether lymphocyte beta-2 receptor number or function reflected receptor status in the lungs and even the early studies suggested that this might not be the case. Galant et al (1978) suggested that reduced neutrophil beta adrenergic receptor number was related to agonist therapy, but Brooks et al (1979) found that a reduced lymphocyte beta receptor number correlated with the disease severity in asthma. In 1980, Galant et al found no differences between untreated asthmatics and normal controls in neutrophil beta receptor numbers or adenylate cyclase responsiveness. There was thus an apparent difference between the changes found on beta-2 receptors on lymphocytes compared with those found on

neutrophils in asthmatic subjects. It was only after the studies of Holgate et al. (1980) documenting development of resistance to intravenous Salbutamol in normal individuals, that differences in the regulation of beta-2 receptors in asthmatics became apparent. Wolfe et al. (1982) studying lymphocyte receptor numbers and isoproterenol stimulation, were unable to find any differences in patients with aspirin induced bronchospasm.

It is now apparent that certain discrepancies in the early studies could have been due to failure to take into consideration normal diurnal variations (Titinci et al. 1984), circannual variations (Haen et al. 1988), age of the patient (Reinhardt et al. 1984) and differences in beta adrenergic receptor numbers in different lymphocyte subsets (Korholz et al. 1988).

It is clear from Table 1 that most investigators have found significantly reduced numbers of beta-2 receptors on lymphocytes in patients with asthma. Lymphocyte beta-2 receptor numbers can be reduced by Salbutamol treatment (Reinhart et al. 1984), Terbutaline treatment (Sano et al. 1984), exercise (Martinson et al. 1985, Reinhardt et al. 1987) and even allergen (house dust mite) challenge (Meurs et al. 1984, 1987 and 1988). It was furthermore shown that atopy itself (Cundell et al. 1984, Gamboa et al. 1987) is associated with a blunted cyclic AMP response in patient's lymphocytes. Reduced receptor responses to catecholamines and lymphocytes have more recently been shown to be reversed by steroid therapy (Brodde et al. 1988) and Ketotifen therapy (Reinhardt et al. 1987, Polson et al.

1988). Reports of impaired adrenaline (but not noradrenaline) secretion (Ind et al. 1985) in acute asthma add further dimension to and are consistent with a disordered beta-2 adrenergic receptor response in asthma. The mechanisms for the reported blunted adrenaline responses have not yet been explained.

Central to any appraisal of the published abnormalities of beta-2 receptor function reported in asthma is that most of the studies showing reduced beta-2 receptor number and function have been conducted on circulating lymphocytes. While the reduced number and function seems to be a consistent finding from the combined published data, there are very papers examining the relationships, if any, between lymphocyte beta-2 receptors and lung beta-2 receptors (Davis et al. 1986, Gamboa et al. 1987). Goldie et al. (1987) have shown a 4-5 fold reduction in relaxant properties of isoproterenol and fenoterol, and have attributed this to reduced smooth muscle beta-2 receptor function, in post-mortem studies of bronchial muscle strip preparations.

Studies in guinea pig models (Barnes et al. 1980), Mu and Bi 1989, Motojima et al. 1989, Gatto et al. 1987) have shown significant decreases in beta-2 receptor numbers in the lungs of asthmatic animals.

In spite of the large number of studies (Table 1) attempting to define the beta adrenergic receptor defect in asthma a number of unresolved questions relating to the theory of beta-2 adrenergic receptor dysfunction in asthma remain.

1. What is the molecular nature and organ distribution of the dysfunction?
2. Is the dysfunction present in all asthmatics or only in a subgroup? (e.g. Atopic or exercise induced asthmatic group)
3. To what extent do genetic factors contribute to the beta-adrenergic receptor dysfunction?
4. Why are the lungs of asthmatics (unlike normals) so susceptible to beta-adrenergic receptor blockade?
5. To what extent do environmental (e.g. drugs, allergens) or other factors (e.g mediators, immunoglobulins) influence beta-2 receptor function in asthmatics.
6. Do the beta-2 adrenergic abnormalities reported *in vitro* (e.g. reduced receptor number or lymphocytes) have clinical significance.
7. Is the beta-adrenergic dysfunction part of a more generalised membrane disorder or related to post-receptor abnormalities?

Novel *in vitro* evidence suggesting that serum factors may directly interfere with catecholamine binding to beta-2 adrenergic receptors was initially advanced in reports by Venter et al. (1980) of autoantibodies to the beta-2 adrenergic receptor and by Fraser et al. (1981) of the association of autonomic abnormalities with autoantibodies to the beta adrenergic receptor in patients with allergic asthma and atopic rhinitis. The initial criteria for the identification of autoantibodies to beta-2 receptors included (1) the ability of serum to affect [^{125}I]-protein A binding to membranes containing beta-2 receptors, (2) the ability of serum to immunoprecipitate solubilised lung beta-2 receptors in an indirect

immunoprecipitation assay (3) the demonstration that the serum factors were immunoglobulins and (4) inhibition of specific binding of [^{125}I]hydroxybenzyl pindolol to canine and calf lung (beta-2) but not to canine heart (beta-1) membranes. Studies attributing the serum effects to the immunoglobulin fraction were however indirect (using anti-human IgG to deplete IgG). It was suggested by these workers that "autoantibodies to beta-2 receptors may mediate a state of beta-adrenergic hyporesponsiveness" which in turn may "exacerbate atopic conditions in much the same manner as the administration of beta adrenergic blocking drugs" (Venter et al 1984). The finding of autoantibodies, using the above criteria, in apparently normal individuals (without atopy or asthma) and in only 10-15% of the atopic patients studied raised questions as to the true significance of the phenomenon in relation to clinical disease and further studies were required to explore the level of interaction with the receptor and to look for functional effects of autoantibodies on human beta-2 adrenergic receptors.

The concept of anti-beta-receptor autoantibodies playing a major role in the pathogenesis of asthma has not gained wide acceptance and since the original reports, Kokubu (1989) and Stiles et al (1984) have stressed the need for further studies. The only subsequent study suggesting that autoantibodies may be of clinical relevance was published by Blecher et al. (1984) and these studies were conducted in collaboration with Venter. (Personal communication).

1.3 Outline of Studies

The relative importance of blockade of beta receptors on smooth muscle compared with the effect of blockade the other cell types is as yet unknown and it is possible that beta adrenoceptor blockade induced by serum factors or mediators may initiate, or sustain, not only the smooth muscle bronchospasm (in acute asthma) but facilitate the increased mucosal oedema, inhibit adrenergic regulation of cholinergic transmission and facilitate the increased mast cell mediator releasability encountered in chronic and more severe forms of asthma.

Based on the foregoing studies and prompted by the need for further studies on the unresolved significance of the effects of serum on ligand binding to beta-2 receptors, I have set up *in vitro* methods to investigate the effects of serum on beta-2 adrenergic receptors. As an initial source of beta-2 adrenergic receptors I chose the guinea pig lung, since this was a well established animal model for asthma and also had a ratio of beta-2:beta-1 receptor of 80:20% which is very similar to the ratio of beta-2:beta-1 found in human lung.

In the first instance, since this field was new to the University of Cape Town, it was necessary to set up reliable ligand binding assays for beta-2 receptors in lung membranes, and to establish a functional assay for beta-2 receptor activity in guinea pig lung tissues. The establishment of these assays is described in Chapter 2.

I then conducted a pilot study, in order to assess whether published reports of autoantibodies may have clinical relevance in the children with asthma attending my allergy clinic at the Red Cross Childrens Hospital, at the University of Cape Town.

These studies confirmed that dilutions of many of the patients' sera did indeed inhibit [^{125}I]-CYP binding to lung membrane beta-2 receptors, that the degree of [^{125}I]-CYP inhibition was variable and in some patients, fairly marked (Chapter 3.2).

Since these initial exploratory studies confirmed a serum inhibitory effect on binding of ligand to beta-2 adrenergic receptors, I embarked upon the more extensive studies reported in this thesis, in order to gain further insight into the origin, regulation and molecular nature of serum and cellular factors which could influence the development, expression and functional integrity of human beta-2 receptors. I have also examined *in vitro* selected biochemical and theoretical genetic models for beta-2 adrenergic receptor dysfunction.

I have started my studies, in Chapter 2, with an original study of the ontogeny of the expression and function of beta-2 receptor in the developing guinea pig foetal lung, and in selected human foetal lungs.

In Chapter 3 the inhibition of ligand binding to guinea pig lung membrane beta adrenergic receptors by human serum was investigated. These initial studies using guinea pig lung membranes indicated that it would be important to obtain a source of human beta-2 receptors to

more adequately and precisely investigate the serum inhibitory effects in further studies, and it was with this intention that I accepted the opportunity to work in the laboratory of Receptor Biochemistry and Molecular Biology at the N.I.H. (Bethesda, Maryland, U.S.A) as a visiting fellow. The original studies which I conducted at the N.I.H. concerning the identification of the beta-2 receptor mRNA and its induction by glucocorticoids and the partial characterization of mutant beta-2 receptors are presented in Chapter 4. On my return to the University of Cape Town I continued with the studies on human serum using cloned, expressed human beta receptors transfected into L cells using the beta-2 receptor gene obtained from the NIH. These studies are presented in Chapter 6. In Chapter 5, I have presented the partial characterization of a monoclonal antibody which I raised to a 20 amino acid peptide derived from a hydrophobic domain of the beta-2 receptor. Studies of rabbit polyclonal antibodies and the immunogenicity of lung membranes are also presented. I have reviewed antibodies raised to beta adrenergic receptors by others. In an attempt to investigate a possible genetic basis for beta adrenergic receptor abnormality in asthma, I have extended the published studies of restriction fragment length polymorphism of the human beta-2 receptor in an attempt to define the site of the polymorphism, and to determine its frequency in asthmatic and normal children living in Cape Town. These studies are described in Chapter 7.

CHAPTER 2

BETA-2 ADRENERGIC RECEPTORS IN THE GUINEA PIG LUNG

2.1 Introduction

The guinea pig is a well described and convenient laboratory model of asthma (Gatto et al. 1987, Goldie et al. 1986, Mu and Bi 1989). Sensitization of guinea pigs with ovalbumin readily leads to severe bronchospasm upon subsequent challenge with ovalbumin (Motojima et al. 1989), which is mediated by anaphylactoid (IgG subclass) antibodies. While there are differences between the guinea pig asthma model and asthma in man, important similarities include the ability to respond with biphasic (late phase) pulmonary responses following allergen challenge (Wieslander et al. 1985) and an almost identical percentage of distribution between beta-1 and beta-2 adrenergic receptors (22:78 in the guinea pig and 30:70 in human lung), with an identical cardioselectivity ratio for beta adrenergic receptor ligands (Engel 1981). An understanding of the normal regulation of beta-2 receptor expression in the guinea pig lung is necessary before perturbations in beta-2 receptor expression in the guinea pig asthma model can be intelligently interpreted.

Since ligand binding assays to beta adrenergic receptors had not been previously performed at the Medical School of the University of Cape Town, my initial project was to set up reliable and reproducible beta receptor ligand binding assays. In this chapter methods for lung

membrane preparation, protein assays, storage and standardization and variables of the beta receptor ligand binding assays and Scatchard plots were evaluated. Alternative methods for improving established assays were also considered.

In order to evaluate the functional capacity of beta-2 receptors in the guinea pig lung I set up a membrane adenylate cyclase assay based on the method of Salomon (1979) and applied both the receptor binding and the functional adenylate cyclase assays to an original study of the ontogeny of the beta-2 receptor in guinea-pig and human foetal lungs.

2.2 Methods

In my exploratory experiments I attempted, as a start, to reproduce the original studies of Venter et al (1980) and Fraser et al (1981) using foetal calf lungs (obtained from the State Abbatoirs, Maitland, Cape Town) and the ligand [^{125}I]HYP (Iodohydroxybenzyl pindolol). I obtained highly variable ligand binding and high levels of non-specific binding to calf lung membranes prepared by the method of Strauss et al (1979), and in view of considerable practical difficulties experienced in obtaining fresh foetal calf lungs from the State Abbatoirs, I discontinued studies with the foetal calf lungs and decided to establish and develop the binding and functional assays in the guinea pig lung, since this was a convenient laboratory model for the study of asthma.

2.2.1 Isolation of cell membranes from guinea pig lungs

The method for the preparation of lung membranes was derived from the method of Strauss et al (1979a), but the protease inhibitors NPGB and EGTA were added and a sucrose phosphate buffer was used for the homogenisation step.

Lungs were removed immediately from adult guinea pigs freshly killed by asphyxiation in CO_2. The trachea and major bronchi were removed and then the lungs were weighed to the nearest 0.01g and washed in homogenization buffer at 4°C (1 mM NPGB,20mM $NaPO_4$, 1mM EGTA in 250mM sucrose, pH 7.2). The lungs were then cut up finely with scissors, immersed in nine volumes of ice cold homogenization buffer and homogenised on ice using a Kinematica PCU-2 Polytron, for two 30 sec bursts. The crude homogenate was transferred into 15ml Sterilin polystyrene conical bottomed tubes and centrifuged in an IEC model PR-6 centrifuge for 8 min at 4°C at 1000g. The supernatant was then decanted into Beckman polycarbonate tubes and centrifuged at 45000g at 4°C for 60 min in a Beckman L8-80 centrifuge, using a 70 Ti model rotor.

After centrifugation, the supernatant was discarded and pellet suspended in 2ml 10% sucrose in 20mM $NaPO_4$, 4mM $MgSO_4$ pH 8.0, loaded on to a 12ml discontinuous sucrose gradient of 20%, 30% and 40% sucrose in 20mM $NaPO_4$, 4mm$MgSO_4$ pH 8.0, and centrifuged at 22000 rpm in a model SW 28 rotor for 80 min, at 4°C, in the Beckman L8-80 centrifuge. Visible bands of lung membranes at the 20%:30% interface

and 30%:40% interface were removed, pooled and centrifuged for 60 min at 100000g at 4°C using the model 70 Ti rotor. The pellet was suspended in 2ml 20mM $NaPO_4$, 4mM $MgSO_4$ pH 8.0, aliquotted into 100 μl volumes, snap frozen and stored in liquid nitrogen.

Protein determination of lung membrane preparations was determined using the commercially prepared Biuret reagent (Worthington).

2.2.2 Beta adrenergic receptor ligands

a) Choice of ligand.

The initial experiments and binding assays were performed with Iodohydroxybenzyl pindolol ([^{125}I]-HYP) obtained from New England Nuclear (Du Pont). [^{125}I]-HYP was obtained with a specific activity of 2200 Ci/mmol. This ligand had been found to be specific for beta receptors by Brown et al (1976) and was used in the studies of Venter et al (1980) and Fraser (1981). However Pocret et al (1978) and Dickinson et al (1981) reported labelling of alpha receptors and serotonin binding sites by this ligand. In view of the reported dual labelling by [^{125}I]-HYP I obtained the new ligand (±)[^{125}I] Iodocyanopindolol ([^{125}I]-CYP) developed by Engel et al. (1981) which has a high affinity for beta receptors (Kd range 27-40 pM) but no affinity to either alpha receptors or serotonin receptors. [^{125}I]-CYP was obtained from New England Nuclear (Du Pont), specific activity 2200Ci/mmol.

b) Supply and storage of ligands.

[^{125}I]-HYP (Cat. no. NEX-125) and [^{125}I]-CYP (Cat. no. NEX-174) were shipped frozen on dry ice in 1-propranalol:water:phenol (50:50:1.2). Ligands were aliquotted in 100μl volumes and stored at -20°C. Prior to use n-propanalol and water were evaporated under dry nitrogen in a gentle stream. A volatile radioiodide safety trap supplied by New England Nuclear was used for the evaporation process.

c) Radiochemical purity.

Radiochemical purity of the iodinated compounds was checked periodically using ascending thin layer chromatography on silica plates using the solvent system methanol:water:acetic acid (90:10:0.15). One microlitre of cold compound (hydroxybenzyl pindolol(HYP) and cyanopindolol(CYP), gift from Dr. Engel,Sandoz Limited, Basel, Switzerland) (1mg/ml), was dispensed on to the lower end of the plate in duplicate. One of the duplicates was spiked with 1μl of the radiolabelled ligands. The plate was then lowered vertically into the solvent and left for 45-60min, after which it was dried at room temperature. Aromatic compounds such as CYP and HYP fluoresced when the plates were placed under an ultraviolet light source. To assess the degree of degradation of the label the "hot" track was cut into 0.5mm strips and counted in the Packard model 3003 gamma counter, (counts at ±70% efficiency). It was found for example that 1μl [^{125}I]-HYP at 74 days from the date of iodination gave 60,000 cpm of which 52 955 cpm moved with the fluorescent band and 5282 cpm

moved with the phenol front. No degradation was found up to 3 months of the labelling date.

2.2.3 [^{125}I]-CYP Binding Assay Protocol

Binding assays were adapted from those of Maguire et al. (1976). For the binding assays 5-10μg of lung membrane protein in 20mM $NaPO_4$ pH 7.2 was incubated with [^{125}I]-CYP in the presence or absence of 10^{-7}M laevopropranalol (a gift from Dr. P. Venables, ICI, Pharmaceuticals Division, Macclesfield, Cheshire, U.K.). The Laevopropranalol hydrochloride (Batch ADM 46051/77 MW 295.8, specific optical rotation -21.73°C) was received as a white crystalline powder.

Incubation of lung membranes was performed in triplicate in a waterbath at 30°C for 30 minutes in polypropylene tubes (Greiner), and the incubation volume was 500μl. The reaction was stopped by addition of 1.25ml 20mM potassium phosphate pH 8.0 containing 1mM magnesium sulphate and 0.1mM ± propranalol (ICI) and filtered immediately through 25mm glass fibre filters (G/F-C). Filters were washed with 25ml of the same buffer at 60ml/min at 30°C and radioactivity of protein bound to the glass fibre filters was counted in a Packard Model 3003 tricarb scintillation spectrophotometer. Changes in specific activity allowing for decay of the [^{125}I] CYP or [^{125}I] HYP were taken into account in the calculations.

Specific binding was calculated by subtracting the counts per minute bound in the presence of 10^{-7}M L(-) propranalol from the counts per minute bound in the absence of L(-) propranalol.

Binding assays (using [^{125}I]-HYP) were initially performed on calf lung membranes, turkey erythrocytes and human lymphocyte preparations, but assays using [^{125}I]-CYP and guinea pig lung membranes were found to be the most convenient, reliable and reproducible. Although a variation in total binding (Bmax) was seen with different guinea pig lungs (range 180-670 fmol/mg) the dissociation constant did not vary markedly between guinea pigs (Kd 26-35pM). In order to eliminate any possible differences in receptor density or Kd in the assays described in Chapter 3, pools of 6-12 guinea pig lungs were prepared on the same day, and stored in liquid nitrogen and served as a standard membrane preparation for the ligand inhibition assays in Chapter 3.

2.2.4 Saturation curves Scatchard plots and Hill plots to the ligand binding of [^{125}I]-CYP to guinea pig lung membranes.

The quantitative approach to drug receptor interaction has its basis in Michaelis-Menten kinetics. According to the law of Mass action, drug-receptor interaction may be represented as follows:

$$R + A \underset{k-1}{\overset{k+1}{\rightleftharpoons}} RA$$

R = receptor
A = drug
RA = receptor drug complex
k+1 = forward rate constant
k-1 = reverse rate constant

At equilibrium velocity of association = velocity of dissociation

$$\frac{(R)\ (A)}{(RA)} = \frac{k-1}{k+1} = Ka$$

Ka = equilibrium constant or association constant

$\frac{1}{Ka}$ = dissociation constant

Saturation isotherms of [^{125}I]-CYP binding to 5-10μg lung membranes were conducted in triplicate using 8-9 increasing concentrations of radioligand (range 10 - 800pM) in the presence or absence of 10^{-7}M (-)L propranalol. A curve of the [^{125}I]-CYP binding to adult guinea pig lung membranes is shown in Fig. 1. Plots of specific binding versus log free ligand were 'S' shaped (Fig. 2) and Scatchard transformation of the data as shown in Fig. 3 (Bmax 670 fmol/mg, Kd 27 pM). Binding was rapid (within minutes) and equilibrium binding reached within 10 minutes was stable for 3 hours at 30°C.

Having established saturation data and Scatchard plots for [^{125}I]-CYP binding to lung membranes, it was necessary to confirm that one molecule of ligand bound one receptor and that there was no co-operativity between multiple receptor sites. This was done by constructing a Hill plot of the data.

A Hill plot (Hill 1909) is constructed by plotting Log (y)/(1-y) on the y axis and Log ligand on the x axis where y is the fraction of the total number of sites bound occupied by ligand molecules. Where approximately 50% of ligand is bound the slope is determined, giving the Hill co-efficient. A Hill co-efficient equal to one implies that there is no co-operativity between receptors, i.e. that the interaction is due to simple Michaelis-Menten kinetics and one receptor binds one ligand molecule.

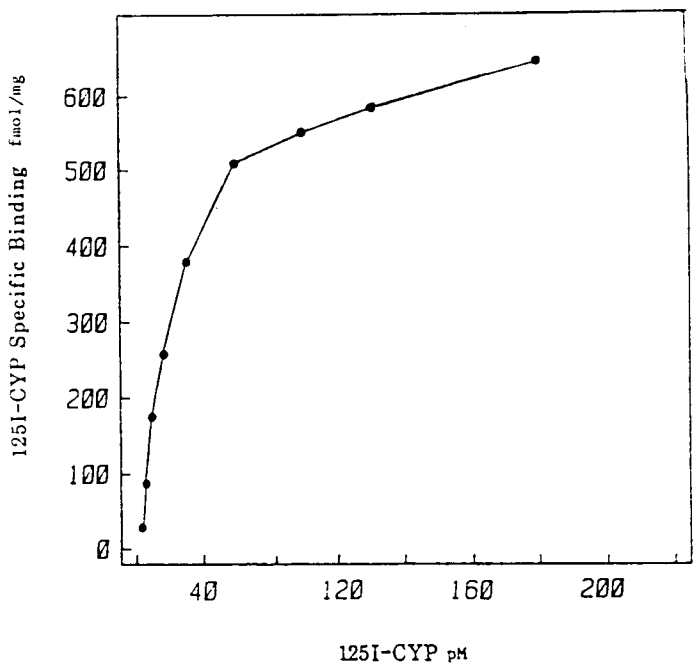


Figure 1. Specific [^{125}I]-CYP binding to 10μg adult guinea pig lung membrane.

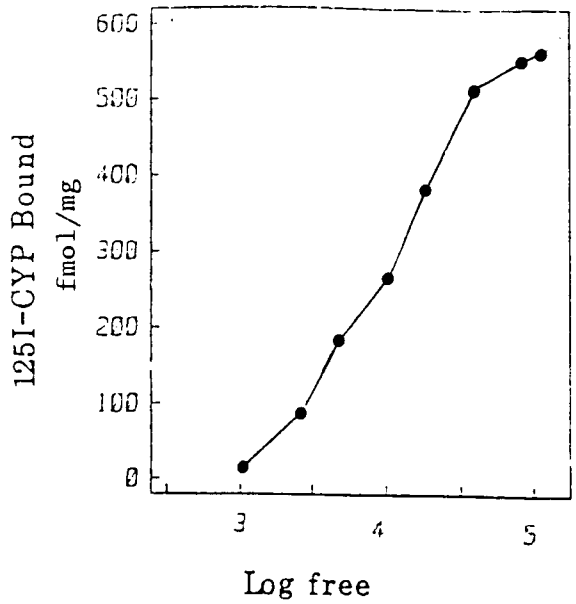


Figure 2. Plot of specific [^{125}I]-CYP binding versus log free [^{125}I]-CYP to adult guinea pig lung membranes (data derived from Fig.1) showing 'shouldered' curve.

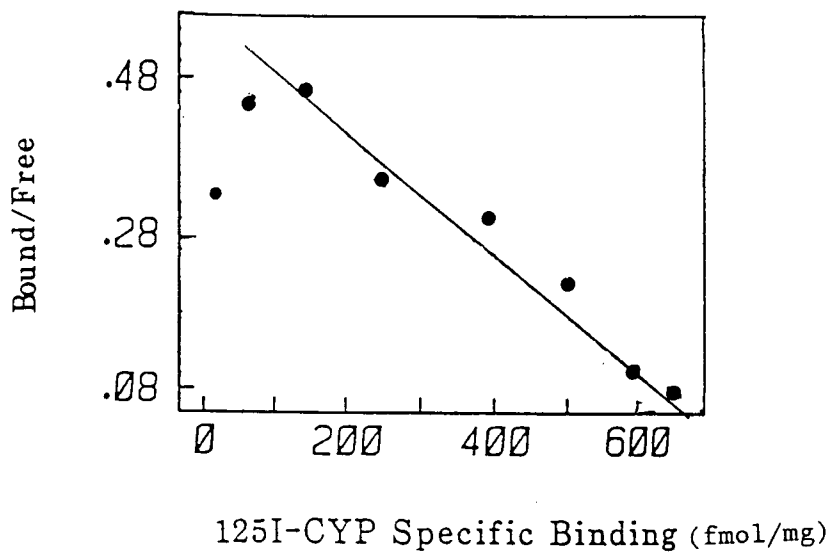


Figure 3. Scatchard transformation of binding of [^{125}I]-CYP to guinea pig lung membranes (data derived from Fig. 1). Bmax 670 fmol/mg; Kd = 27pM.

Fig. 4 shows a Hill plot using data obtained for binding of [[^{125}I]-CYP to guinea pig lung membranes in a saturation experiment on which Bmax= 670 fmol/mg.

2.2.5 Beta receptor subtypes in the guinea pig lung membrane

In order to confirm that both beta-2 and beta-1 receptor subtypes were measured in the guinea lung membrane [^{125}I]-CYP binding assay, the method of Engel (1981) was employed.

ICI 118-551 a potent, highly beta-2 selective adrenoceptor antagonist (O'Donnel and Wonstall 1980) (erythro-DL-1)(7 methylindan-4-yloxy)-3 isopropyl aminobutan-2-ol) was a gift from Dr. P. Venables ICI Pharmaceuticals Division, Macclesfield, Cheshire.

The ring structure in ICI 118-551 is unique among beta-2 selective antagonists and must also be important for selectivity, since it is the only structural feature which distinguishes it from the less selective alpha-methyl propranalol. (O'Donnel and Wonstall, 1980). Using this ligand Nahorski and Barnett (1981) showed that beta-1 and beta-2 adrenoceptors can co-exist in tissues in a non-interacting manner and are probably quite separate entities.

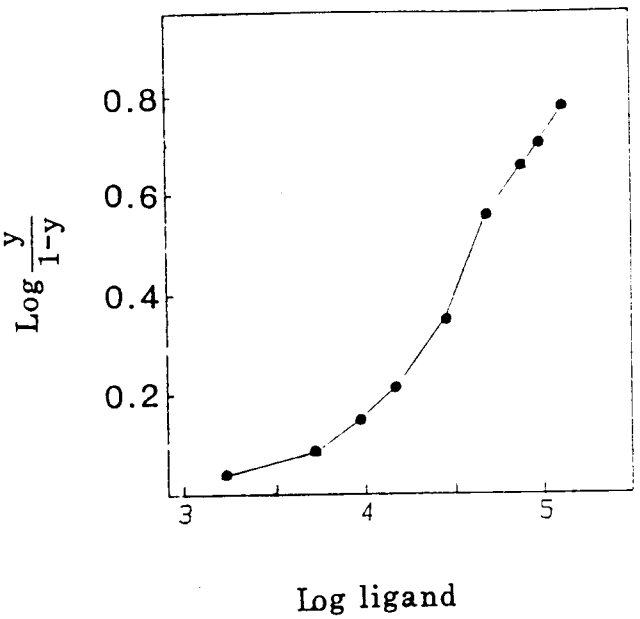


Figure 4. Hill plot of the binding data from Fig. 1 where y = the fraction of the total number of sites bound by [^{125}I]-CYP.

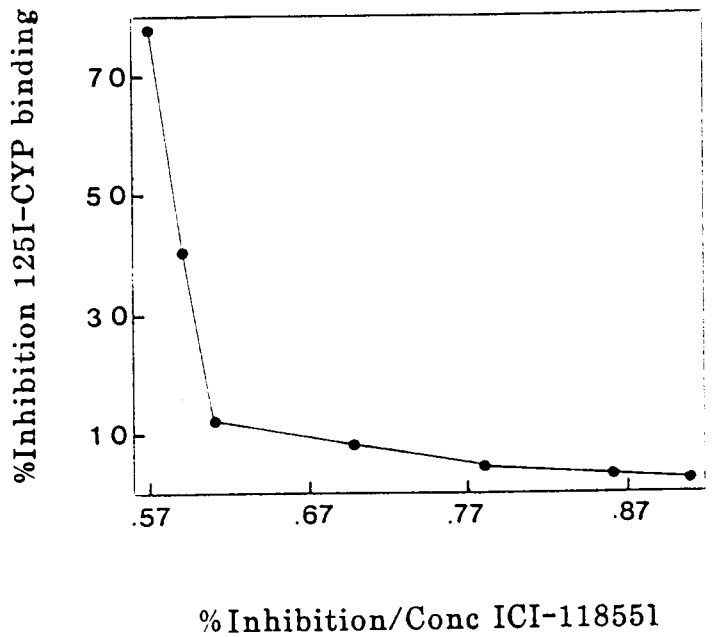


Figure 5. A non-linear pseudo Scatchard or Hofstee plot using the beta-2 selective ligand ICI 118, 551, demonstrating that both beta-1 and beta-2 receptors in the guinea pig lung membrane preparation are labelled by [^{125}I]-CYP.

Several investigators have attempted to use receptor binding assays to classify beta adrenergic subtypes (Rugg 1978, Minneman 1980). All methods so far in use involve examination of the characteristics of displacements of a radioligand by a drug which shows in vitro selectivity for beta-1 or beta-2 receptors.

Fundamental assumptions underlying available methods for measuring beta adrenergic receptor subtypes with radioligand binding assays have been outlined by Minneman and Molinoff (1980) as follows:

1. There are only two beta adrenergic subtypes in the tissues being examined.
2. The interaction of agonists and antagonists with each receptor subtype follows simple Michaelis-Menten Kinetics with Hill coefficients of 1.0.
3. The radioligand has the same affinity for both receptor subtypes.

Using the highly selective ligand such as ICI 118,551 to inhibit radioligand binding by [^{125}I]-CYP and plotting the inhibition in a modified Scatchard, or Hofstee plot (Fig. 5), a biphasic graph confirmed that the guinea pig lung membranes which I had prepared have two subclasses of beta receptors. Engel (1981) has previously shown that beta-2 adrenergic receptors predominate over beta-1 receptors in the guinea pig lung.

Analysis of non-linear Hofstee plots is preferably performed by the computerised procedure described by Minneman et al (1979). Hancock et al

(1979) have however applied the computerised non-linear least squares curve fitting techniques of Feldman (1972) to quantitate receptor subtypes since these methods are more accurate than graphical methods such as the Hofstee plot. The main objection to the use of the Hofstee plot is the nature of data transformation techniques in which numbers subject to experimental error are divided by the low concentrations of competitor to produce graphical co-ordinates. These transformed values are exceedingly sensitive to small errors in the data obtained and can produce large fluctuations in the shape of the curve.

More simply the formula of Engel (1979) may be applied to competition curves for binding

$$RA = \frac{RT}{1+\frac{Ka}{A} \cdot 1 + \frac{B}{Kb}}$$

RA = Concentration of bound ligand under steady state

R = Free concentration of receptor

a = Concentration of radioligand A

RT = Total receptor concentration

b = Selective beta blocking agent

Ka = Dissociation constant of A

Kb = Dissociation constant of B

In this equation radioligand A binds with equal affinity to beta-1 and beta-2 receptor subclasses.

In the experiments to study the stereoselectivity of the binding sites in the guinea pig lung membranes by competition with the beta-2 selective ICI 118-551 and beta non-selective L(-)propranalol the inhibition constants Ki (Ki = EC 50%) obtained were as follows:

Ki of ICI 118,551 = 2.6×10^{-8}M

Ki of L(-) propranalol = 1.32×10^{-9}M

The theoretical Ki for L(-)propranalol to glioma membrane beta receptors is 1.2×10^{-9}M (Engel 1981).

The displacement curve for L(-) propranalol was uniphasic whereas a flatter and shallower curve obtained with ICI 118,551 was consistent with the presence of beta-1 and beta-2 receptors in the membrane preparation.

The Kd of the competing ligand may be obtained by applying the Prussoff equation.

$$Kd = \frac{EC\ 50}{\frac{S}{km} + 1}$$

S = Concentration of [^{125}I]-CYP
km = Kd for [^{125}I]-CYP
EC50 = Concentration of competing ligand at 50% inhibition of binding.

Thus in the above experiment where inhibition studies were performed at the Kd of [^{125}I]-CYP, Kd = EC 50/2

Kd ICI 118,551 = 1.3×10^{-8}M

Kd L propranalol = 0.66×10^{-9}M

These data confirmed that I had established an assay for guinea pig lung membrane beta-2 receptors which was consistent with expected values.

2.2.6 Specific binding of [^{125}I]-CYP to different fractions of the lung membrane preparation.

Binding assays were performed at the Kd of [^{125}I]-CYP on lung preparations at several stages of the extraction to determine which fraction had the greatest density per 50μg protein, to assess the extent of beta receptor loss during extraction, and to investigate an alternative method of preparing receptors in cell membranes, namely, dismembranation. Guinea pig lungs were snap frozen in liquid nitrogen and then dismembranated using a Brawn Micro dismembranator type 853062 prior to homogenizing in order to facilitate even homogenization of the lungs and possibly reduce the time of homogenization. Homogenization with the PCU-2 Kinematica Polytron homogenizer was found to raise the temperature of membranes above 4°C. Results of this assay performed in triplicate (at the Kd 27pM [^{125}I]-CYP) are shown in Table 5.

Table 5. Specific binding of [^{125}I]-CYP to preparative fractions of beta-2 receptors with or without dismembranation.

Lung fraction	Specific Binding of [^{125}I]-CYP/50μg protein	
	Without dismembranation (Mean cpm)	With dismembranation (Mean cpm)
Crude Homogenate	866	413
50000g pellet	2953	1074
Sucrose gradient pellet	1255	407
Sucrose gradient 30:40 interface	4207	2389
Sucrose gradient void	490	39

This experiment confirmed that the best yield of beta receptors was obtained at the 30:40 sucrose gradient interface, as expected, but that dismembranation reduced the number of receptors recovered by approximately 50%. This reduction is probably due to the additional freezing and thawing required for dismembranation. Noteworthy is the significant amount of [^{125}I]-CYP binding discarded in the sucrose gradient pellet, possibly due to incomplete homogenization. These experiments confirmed that homogenization as applied to the preparative method of Straus et al (1979) was superior to dismembranation.

2.2.7 The effect of storage on ligand binding.

(i) Liquid nitrogen, 4°C, -20°C and room temperature.

Storage conditions were studied as follows:-
Lung membranes were stored at 4°C, room temperature, -20°C and in liquid nitrogen. Specific [^{125}I]-CYP binding was compared on membranes stored at different temperatures for varying time intervals.

Liquid nitrogen storage resulted in the highest [^{125}I]-CYP binding values which were reproducible for several months. Storage at 4°C overnight did not result in loss of radioligand binding but, storage at -20°C overnight (which involved freezing and thawing) resulted in loss of radioligand binding. Storage at room temperature resulted in an appreciable loss of radioligand binding.

(ii) Glycerol-sucrose cryopreservation.

Since storage facilities in liquid nitrogen were limited, in order to store batches of guinea pig lungs, the cryopreservation method published by Crawford et al. (1984) was investigated. Dissected fragments of lung tissues obtained freshly were immediately immersed in 3mM $MgCl_2$, 20mM Hepes in 0.5M sucrose pH 7.4, mixed with an equal volume of glycerol and stored at -20°C. No loss of radioligand binding to membranes of lungs stored in glycerol-sucrose was detected in the first two months of storage. However, assays performed on lungs after two months storage showed a fall off in radioligand binding. For the

studies reported in this Chapter and Chapter 3, lung membranes were prepared freshly and stored in liquid nitrogen.

2.2.8 Investigation of an alternative method for separating bound from free [^{125}I]-CYP

Using the binding assay of Maguire et al (1976), it was found that the volume of 20mM K_2HPO_4 buffer used to wash the Whatman GF-C filter paper influenced the amount of bound [^{125}I]-CYP that was detected. This is illustrated in the following data set:

100μg guinea pig lung incubated for 30 min with 25000 cpm [^{125}I]-CYP

Volume K_2HPO_4 wash	Mean cpm on filter (triplicate)
no wash	12500
15 ml	4039
30ml	2464
60ml	2024

In order to minimise the possibility of non-specific binding to the GF-C filters and the need to wash each filters with relatively large volumes of wash buffer, the method of Penefsky (1977), using Sephadex G50 columns was investigated as an alternate method for separating membrane bound from free [^{125}I]-CYP. The binding assay volume (in 2.2.3) was scaled down to 100μl total volume, since a volume not greater

greater than 100μl can be loaded on to the Sephadex G50 column in a Tuberculin syringe. Since this method would require less receptor and ligand (one fifth) it seemed worthwhile to investigate fully.

125μl of the assay binding mixture (as in 2.2.3) was incubated for 30 min at 30°C in a water bath, after vigorous mixing on a vortex, 100μl was then loaded on to Sephadex G50 columns which had been prepared as follows:-

Sephadex G50 fine was swollen in twice the bed volume of the incubation buffer (Na_2HPO_4 pH 7.4) overnight and suspended by gently stirring with a magnetic stirrer. 1 ml of Sephadex G50 suspension was pipetted in to a 1ml Tuberculin syringe (Becton Dickinson) which had been fitted with a 5mm diameter polyethylene disc at the base, previously soaked in 0.1% Triton in incubation buffer, and centrifuged in a 15ml Sterilin test tube in a PR6 centrifuge at 800 rpm for 2 minutes. The Sephadex G50 gently packed by this procedure occupied about 50% of the height of the syringe.

In preliminary experiments investigating the columns using iodinated sheep or mouse gammaglobulin, a 97% recovery obtained if the polyethylene discs had been soaked in 0.1% Triton. If dry polyethylene discs were used (i.e. not previously soaked in 0.1% Triton) the recovery was only 75%, suggesting that polyethylene discs not treated with 0.1% Triton could trap protein and reduce the recovery. In order to determine how much guinea pig lung membrane is retained in Sephadex G50 columns, lung membranes were iodinated by the

Lactoperoxidase method (Appendix A) prior to spinning through Sephadex G50.

Lactoperoxidase labelled membranes were loaded on to Sephadex G50 columns, eluate collected and reloaded on to fresh Sephadex G50 columns 3 times. It was found that each time the labelled membranes were spun through the Sephadex G50 columns approximately only 50% of the counts were recovered in the eluate. Data from this experiment is shown in Appendix A1.

It was found that incubation of the membranes at 4°C with 0.1% Triton 100 prior to spinning through the Sephadex columns increased the specific binding recovered by 100% without increasing non-specific binding. This effect was probably due to the solubilising effect of the Triton removing the receptors from the membranes. The method of Penefsky for separating bound from free ligand, although theoretically attractive, was therefore not applicable for membrane preparations and I therefore used the Whatman G/FC filters.

2.2.9 The effect of magnesium concentration on the [^{125}I]-CYP binding assay.

4mM $MgSO_4$ was used in the [^{125}I]-CYP binding assays on calf lung membranes by Maguire et al.(1976). I examined the effect of varying concentration of Mg^{++} ions on the [^{125}I]-CYP binding assay using guinea pig lung membranes to determine how sensitive the ligand binding was to

pig lung membranes to determine how sensitive the ligand binding was to changes in the Mg^{++} concentration. 50μg guinea pig lung membrane protein was incubated with [^{125}I]-CYP at the Kd in the presence of concentrations of Mg^{++} ions between 1-100mM. No differences in specific binding were observed between 1.0-15mM Mg^{++} but concentrations between 20mM and 100mM inhibited 25% of the binding. Venter (personal communication) has also found that although some Mg^{++} is necessary in the binding assay, a fairly wide range of concentrations is permissible. My findings would support this observation.

2.2.10 Effect of protease inhibitors

Recent studies have alluded to the importance of proteases in decreasing receptor number. Using the fluorometric assay of Heussen et al., (1984) which measures the ability of proteases to cleave an artificial substrate, CBZ-gly-gly-arg-AMC* by following the increase in fluorescence (measured over 5 min) as the Arg-Amc bond is cleaved by the protease, it was found that the guinea pig lung membrane preparation had an average protease activity of 156.8 Fluorimetric units/ml compared with a protease activity of 25 Fluorimetric units/ml in normal plasma. Various protease inhibitors were investigated. Protease activity in both guinea pig and calf lung membrane preparations was completely inhibited by 1mM NPGB. Concentrations of NPGB up to 10mM and 1mM EGTA in the homogenization buffer did not reduce ligand binding to guinea pig lung membranes. In view of the fact that 1mM NPGB did not influence ligand binding directly, I added 1mM NPGB and EGTA to the homogenization buffer, when membranes were isolated from the guinea pig lungs for ligand binding assays.

2.2.11 Photo affinity labelling of guinea pig lung beta-receptors with (±)-3-[^{125}I] iodocyanopindolol-diazirine

Since it would be convenient if beta receptors in guinea pig lung membranes could be covalently labelled, so that receptors which have had been immunoprecipitated by antibodies could subsequently be identified by autoradiography, labelling with a carbene generating photo-affinity probe for beta adrenergic receptors was investigated.

(±)-3-[^{125}I] Iodocyanopindolol-diazirine has been synthesised by Burgermeister et al. (1983). The long wavelength absorption of the diazirine permits formation of the carbene by photolysis under very mild conditions. [^{125}I]-CYP-diazirine was previously shown to label at least 5 proteins in guinea pig lung membranes of which one with approximate MW 67 000 was labelled specifically.

Method of Photolabelling

The method of Burgermeister (1983) was followed. Briefly 5 mg guinea pig receptor protein was incubated in the dark at 30°C for 60 min in 2 ml 10mM Tris-HCl pH 7.4 (which had been nitrogen saturated) with 1nM [^{125}I]-CYP diazirine, in the presence or absence of 10^{-5}M propranalol.

At the end of the incubation, samples were cooled on ice for 10 min. made up to 6ml with ice cold 10mM Tris HCl pH 7.4 and purged with nitrogen for 15 seconds in the dark.

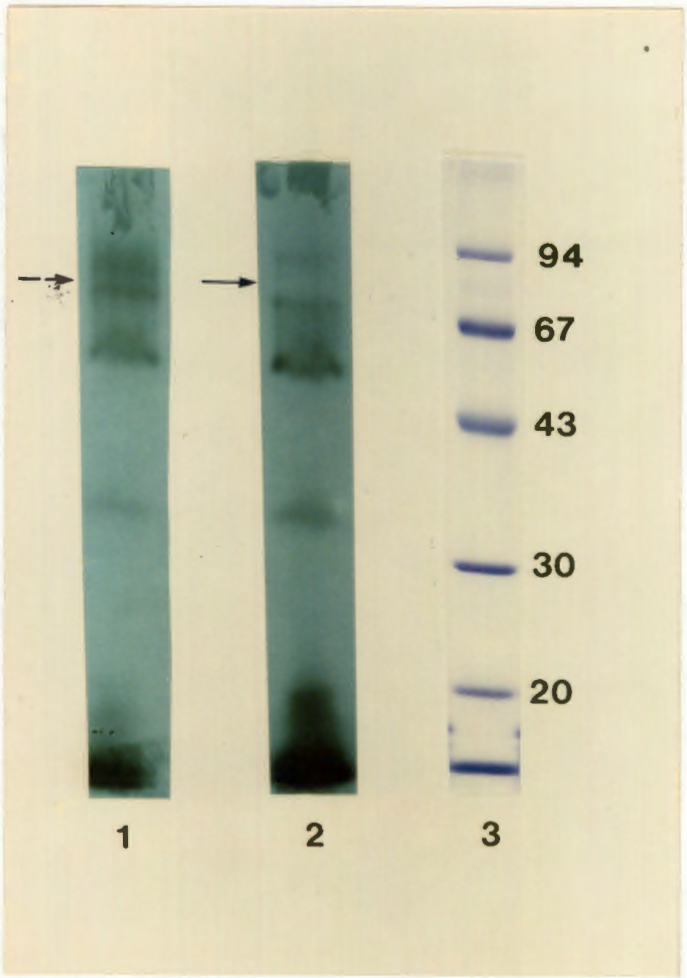


Figure 6. Autoradiograph of 11% polyacrylamide gel electrophoresis of guinea pig lung membranes labelled with [^{125}I]-CYP-diazirine in the absence (left lane) or presence (middle lane) of 10^{-5}M L(-)propranalol. Molecular weight markers are shown on the right.

Photolysis was performed using a Quartz GES m.b.H. type 5241 U.V. lamp placed 8-10 cm above the incubation tubes for 60 min. Guinea pig lung membranes were then pelleted at 30 000g for 20 min at 4°C, washed with 10mM Tris-HCl pH 7.4 at 30 000g for 20 min at 4°C. 0.5ml Triton in 20mM sodium phosphate pH 7.4 was added to each tube and the tubes gently shaken for 60 min. 80μl of this suspension plus 20μl of sample buffer (312.5mM Tris-HCl pH 7.4,10% SDS,50% glycerol,25% 2-mercapto ethanol,0.005% Bromophenol blue pH 6.8) was mixed and loaded to an 11% polyacrylamide gel with molecular weight markers (Pharmacia) and electrophoresed for 8 hours in an LKB vertical electrophoresis unit. After staining the gel with Coomassie blue, destaining and drying on filter paper, autoradiography was carried out at -70°C using a Kodak Xray film in casettes with intensifying screens. Exposure time was 10 days.

Prior to photolabelling experiments, thin layer chromatography of [^{125}]-ICYP-diazirine in 90% chloroform 9% methanol 1% acetic acid demonstrated that 99% of the photo-affinity reagent moved as a solid front. Initial experiments also showed that 1nM [^{125}I]-CYP-diazirine bound to albumin on exposure to U.V. light and that this was not inhibited by the presence of 10^{-5}M L(-) propranalol. [^{125}I]-CYP-diazarine was also found to bind to other human serum proteins.

Photolabelling of guinea pig lung membranes with [^{125}]-ICYP-diazirine (Fig. 6) shows a broad band of labelling between 67 kDa and 94 kDa which was inhibited by 10^{-5}M L(-) propranalol, corresponding to the molecular weight for the beta adrenergic receptor in the guinea

pig. [^{125}I]-CYP-diazirine also labelled at least five other proteins in the guinea pig lung membranes which are not inhibited by 10^{-5}M L(-)propranalol. Photolabelling was also applied to mouse L cells in studies of glycosylation mutants of the beta-2 receptor discussed in 4.3.5.

2.2.12 Establishment of a functional assay for adenylate cyclase activity in guinea pig lung homogenates.

With the view to investigating possible effects of human serum fractions on beta-2 adrenergic receptor function and to study developmental changes in adenylate cyclase activity, a functional adenylate cyclase assay based on the method of Salomon et al (1979) was established for the guinea pig lung homogenates.

This functional assay for adenylate cyclase is based on the fact that adenylate cyclase (ATP pyrophosphate-lyase) catalyses the conversion of ATP to 3'5'-cyclic AMP and pyrophosphate in the presence of GTP and magnesium ions, when activated by hormone.

$$\text{Adenosine 5'- *P-P-P} \xrightarrow[\text{GTP},Mg^{2+}+\text{ hormone}]{} \text{adenosine 3'5'- P* + P-P}$$

Difficulty in assaying this enzyme in vitro results from the fact that it is present in the membrane preparation in very small amounts, so that only a minute fraction, usually less than 0.05% of the substrate ATP undergoes cyclization to form cyclic AMP. Assay sensitivity and reliability is dependent upon and proportional to the efficiency with

which cyclic AMP is separated from ATP and its breakdown products. Separation of the reaction product is achieved by sequential chromatography on Dowex 50 cation exchanger and on neutral alumina.

Adenylate cyclase assay protocol.

Radioactive Chemicals used:

1. Adenosine 3'5' cyclic phosphate ammonium salt [2,8-^{3}H] specific activity 31.5 Ci/mmol Lot 1763-165 (NET-275)
2. Adenosine 5'-Triphosphate, Tetra (triethylammonium) salt [α-^{32}P] specific activity 760 Ci/mmol. Lot 2102-208 (NEG 003X)
3. Adenosine 5'-Triphosphate, Tetrasodium salt [U-^{14}C] specific activity 593mCi/mmol Lot 1827-117 [NEC 417]

Radiochemical purity of [U-^{14}C]ATP and [2,8-^{3}H] cAMP was checked periodically using isobutyric acid:ammonium hydroxide: EDTA (0.1M): water (66:1:1:32) and isopropanol: ammonium hydroxide: water (7:1:2) on Whatman No. 1 paper, descending, respectively.

The incubation mixture used in the adenylate cyclase assay was based on the method of Whitsett et al. (1981) for rat lung homogenates and was made up as follows:-

For 10 assays:

Reagent	Vol μl	Final Concentration
1M Tris-HCl pH 7.6	100	100mM pH 7.4
42mM $MgCl_2$	100	4.2mM
125mM Sucrose	100	12.5mM
0.5mM EGTA	100	0.05mM
100mM ATP	10	1 mM
10mM cAMP	100	1 mM

Creatinine phosphate (Boehringer) 2.7mg and Creatinine phosphokinase 0.125mg (Boehringer) was added to the above mixture (510μl) as an ATP regenerating system. Activity of the Creatinine phosphokinase was 860 units/mg dry compound.

50μl of above reaction mixture is added to sodium fluoride (NaF), Isoproterenol, guanosine triphosphate (GTP) or Gpp(NH)p in distilled water, crude homogenate (20 μg) (which had been prepared in 50mM Tris-HCl (pH 7.4) 10mM $MgCl_2$) and [α^{32}P] ATP or [U-^{14}C] ATP 0.1μCi to a final volume of 100 μl. After incubating for 15 min at 30°C, 100 μl stopping solution (2% sodium lauryl sulphate, 45mM ATP, 1.3mM 3'5'-cyclic AMP) was added and after adding the stopping solution, 50 μl [^{3}H] AMP(± 20 000 cpm) tracer, (volume now 250 μl) was added. The test tubes were placed into a boiling water bath for 3 min and then allowed to cool to room temperature, to allow complete solubilization of all the membrane proteins. Thereupon 0.8 ml double distilled, deionised H_2O was added and the final mixture was decanted on to freshly washed Dowex AG 50W-X4 columns, the column was washed twice with 1 ml H_2O, and allowed to drain (to elute [α-^{32}P] ATP).

The Dowex columns were then placed over a rack containing alumina columns, (prepared as in Appendix B), freshly washed with 0.1M imidazole pH 7.3, and 3 ml H_2O was added on to Dowex columns and effluent allowed to drain completely through both columns into the collecting tray. (In this step the cyclic AMP was eluted from the Dowex columns and is adsorbed quantitatively on to the alumina columns).

The rack containing the alumina columns was placed over scintillation vials filled with 4 ml scintillation fluid. 4 ml of 0.1M imidazole buffer pH 7.3 is added to each column and the effluent was allowed to drain completely from all the columns (eluting the 3':5'-cyclic AMP into the scintillation vials).

Scintillation vials were capped, shaken vigorously for a few minutes and counted on a Beckman LS 3800 using $^{32}P/^{3}H$ or $^{14}C/^{2}H$ dual label counting programmes. Cyclic AMP formed was determined by converting the cpm in the ^{32}P or ^{14}C channel to pmoles/15min/mg membrane allowing for the recovery of [^{3}H]-cAMP (usually 60-80%).

2.2.13 Isoproterenol, forskolin and sodium fluoride stimulated adenylate cyclase activity in guinea pig lung homogenates.

Before assessing functional effects of development, or serum factors on guinea pig lung beta-adrenergic receptors, it was important to document the normal activation of adenylate cyclase in the system I had set up. Assays for adenylate cyclase activity were performed on crude homogenates of fresh lung specimens. Briefly lungs were removed by

dissection, cut into small pieces with scissors and homogenised in 9 volumes of ice cold homogenisation buffer (50mM Tris-HCl pH 7.4 10mM $MgCl_2$) on ice using a kinematica PCU polytron for 2 x 30 sec bursts. The crude homogenate was transferred to 15 ml sterilin polystyrene conical bottomed tubes and centrifuged in an IEC model PR-6 centrifuge for 8 min at 4°C at 1000g. Supernatants from this centrifugation were assayed in duplicate or triplicate for adenylate cyclase activity.

In comparative experiments, using 5 μl crude guinea pig lung homogenate (approx. 20μg protein), 10^{-5}M Isoprenaline, 10^{-5}M GTP or 10mM NaF, I found that the percentage stimulation of adenylate cyclase activity in this system, as measured by cyclic AMP recovery from labelled substrate, was equal when either [α-^{32}P] ATP or [U-^{14}C] ATP was used as substrate (Fig. 7). Thus, for example, using fresh guinea pig lung homogenates in a comparative experiment, the mean % of stimulation of adenylate cyclase activity above basal levels with [α^{32}P] ATP as substrate for 10mM NaF was 576%, for 10^{-5}M GTP 81%, for 10^{-6}M isoproterenol 42% , 10^{-6}M isoproterenol and 10^{-5}M GTP 148%. Using [U-^{14}C] ATP for 10mM NaF was 571%, for 10^{-5}M GTP 52%, for 10^{-6}M isoproterenol 38% and 10^{-6}M isoproterenol and 10^{-5}M GTP 121%. Since it was more economical to use the more stable [U-^{14}C]ATP (in view of the very short half life of [α-^{32}P] ATP), [U-^{14}C] ATP was used in the definitive experiments of guinea pig lung development (in 2.3.3).

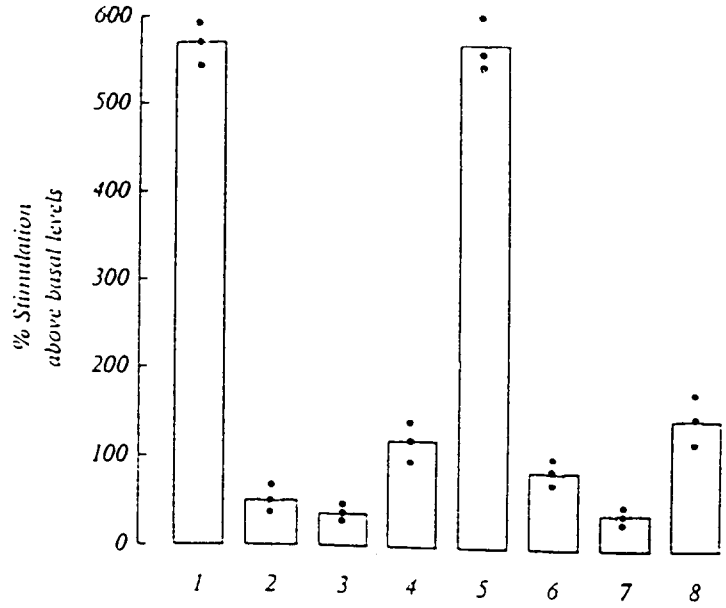


Figure 7. Comparison of the % stimulation of guinea pig lung crude homogenate adenyl cyclase measured by cAMP production (Salomon 1979) above basal levels with 10mM sodium fluoride (1,5), 10^{-5}M GTP (2,6), 10^{-5}M(-) isoproterenol (3,7) and 10^{-5}M GTP with 10^{-5}M (-)isoproterenol (4,8), using ^{14}C(U)ATP (Bars 1-4) or α^{32}pATP substrates (Bars 5-8). Bars represent mean of triplicate experiments (•).

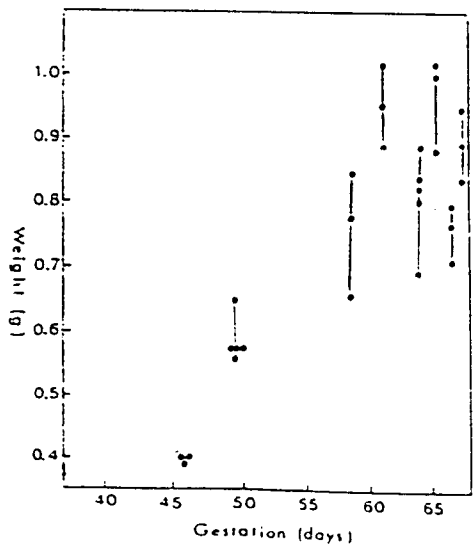


Figure 8. Gestational plot showing variability of mean lung weights (left and right) of foetal guinea pigs within each litter. (•) represents each foetal lung pair weighed).

2.2.13(i) Effect of protease inhibitors

Although 1mM NPGB was an essential protease inhibitor which had no effect in the [^{125}I]-CYP binding assays, it was found that 1mM NPGB markedly inhibited Na fluoride and isoproterenol stimulated adenylate cyclase activity but not basal activity in the crude homogenates. Homogenates were therefore prepared in 50mM Tris HCl 10mM $MgCl_2$ pH 7.4 without protease inhibitors and used freshly.

2.2.13(ii) Effect of Guanosine triphosphate

Concentrations of GTP greater than 10^{-4}M inhibited adenylate cyclase activity to below basal levels. However, activation of adenylate cyclase stimulation was observed in the presence of 10^{-5}M GTP and produced a synergistic effect with 10^{-5}M isoproterenol.

The synergistic effect on adenylate cyclase stimulation when GTP (10^{-5}M) was added to isoproterenol 10^{-5}M (or 10^{-6}M) was highly reproducible. Activation of adenylate cyclase by GTP in the absence of hormones previously described by Lad et al. (1984) in neutrophil membranes prepared by gas cavitation, was indicative of a state of receptor cyclase uncoupling, while membranes from sonicated cells displayed normal GTP dependent coupled activation by isoproterenol. Crude homogenates used in my assays clearly display both patterns, suggesting that a mixed population of coupled and uncoupled receptors

exists in these homogenates and that no conclusions should be drawn as to the overall state of coupling in assays of crude homogenates.

2.2.13(iii) Effect of storage

Fresh crude homogenates were used for the adenylate cyclase assays since it was found that adenylate cyclase activity was markedly depressed by freezing and thawing. (Guinea pig lungs which had been stored in glycerol sucrose at -20°C retained good basal activity and sodium fluoride stimulatable activity, but isoproterenol stimulated activity was variable i.e. normal in some samples but depressed in others).

2.3 Functional and numerical ontogeny of beta receptors in the developing guinea pig foetal lung.

The normal ontogeny of the expression of beta adrenergic receptors and their ability to couple with adenylate cyclase via the G proteins has been reported in the foetal rat (Whitsett et al. 1981), and in the foetal rabbit it has been reported that glucocorticoids increase pulmonary beta-adrenergic receptors and surfactant production at term (Cheng et al. 1980). Little is known about the normal expression and function of the beta adrenergic receptors in the foetal guinea pig. Using a Medline search, I could find only one reference referring to developmental changes in the guinea pig lung beta receptors and this study merely compared newborn with adult guinea pig lungs (Gatto et al. 1984). I considered that it would be worthwhile to apply the

techniques for receptor measurement and adenylate cyclase activity which I had established, to conduct an original study of the developmental expression of the guinea pig lung beta receptors during foetal life. I reasoned that identification of a well defined gestational period of rapid beta-2 receptor expression, would be extremely useful for future studies of the genetic, hormonal or biochemical factors responsible for inducing and sustaining normal beta-2 receptor expression in the guinea pig lung.

2.3.1 Breeding Colony

A breeding colony involving 80 guinea pigs was established. Females with estrus were selected by checking for a perforated vaginal membrane and lordosis, separated from the colony and mated in the dark, noting the date of the mating. Successful mating was confirmed by checking for a vaginal plug and these females were then transferred to individual cages. Using this system 10 out of 40 matings resulted in pregnancy and lungs from litters of guinea pig foetuses obtained at 10 points between 24 and 68 days were studied.

Pregnant females were killed by CO_2 asphyxiation. Maternal lungs and the lungs of the fetuses were removed and crude homogenates and membranes were prepared from the lungs according to the method described in 2.2.1. Mean weights of the left and right foetal lungs were recorded and plotted as a function of gestational age. The right lungs were used in the functional adenylate cyclase assay and beta adrenergic receptor number was measured by Scatchard analysis of the

saturation binding of [^{125}I]-CYP to maternal or foetal lung membranes prepared from the left lungs.

2.3.2 Foetal lung weight as a function of gestational age.

The average size of the guinea pig litters was 3 foetuses (range 2-6 foetuses). A wide range of variability in the weight of the lungs of litters of the same gestational age was noted (Fig. 8). Even within a litter there was sometimes one foetus whose lungs weighed markedly less than the others. The left foetal lung always weighed less than the right foetal lung. There was also a variation in the wet weight of the maternal lungs (mean maternal right lung 2.3g (range 1.58-3.3g; mean maternal left lung 1.95 (range 1.53-2.15)).

2.3.3 Beta adrenergic receptor expression in the developing foetal guinea pig lung.

[^{125}I]-CYP binding assays were performed on guinea pig foetal lungs to study the developmental expression of beta-2 receptors, and to identify periods of increased receptor expression for future biochemical and mRNA studies.

Lung membranes were prepared from the maternal and foetal guinea pigs at ten gestational time points from 23 days gestation to day 1 post-natally. Maternal [^{125}I]-CYP binding data at each gestation point served as an internal reference of acceptable binding for each assay.

Adenylate cyclase assays were performed on fresh homogenates from the same maternal-foetal pairs used in the [^{125}I]-CYP binding assays (2.3.4). Saturation curves were performed on pooled foetal lungs at each gestational point studied and Scatchard plots of the binding data were used to determine the maximal binding (Bmax) and ligand affinity (Kd) at each point. Binding data has been plotted using Enzfitter non-linear iterative regression data analysis programme for IBM PC designed by R.L. Leatherbarrow, Department Chemistry, Imperial College of Science and Technology, South Kensington, London.

Saturation curves of [^{125}I]-CYP binding to foetal guinea pig lung membranes at 23 days, 42 days, 46 days, 47 days, 49 days and 53 days (Fig. 9) showed low dormant levels of beta-2 receptor expression with a Bmax of between 20-40 fmol/mg lung membrane protein (on Scatchard plot). However, between day 53 and day 62, induction of beta-2 receptor expression occurred. The levels of expression in foetal lungs assayed at 5 points after day 62 were variable, but were approximately half the values found in the adult lungs (mean adult binding 425 fmol/mg range: 380-500fmol/mg protein) Fig. 10.

Some guinea pigs gave birth at 64 days whereas others had not delivered by 65 days gestation. Scatchard plots of [^{125}I]-CYP binding to foetal lungs earlier in gestation showed similar affinity (Kd 27-35pM) of [^{125}I]-CYP binding for the beta-2 receptors found in maternal lungs (Kd 30pM). Scatchard plots and saturation curves of the binding of [^{125}I]-CYP to guinea pig foetal lungs early, (49 days), and at the end (64 days) of the gestation period, are shown in Fig. 11 and Fig. 12.

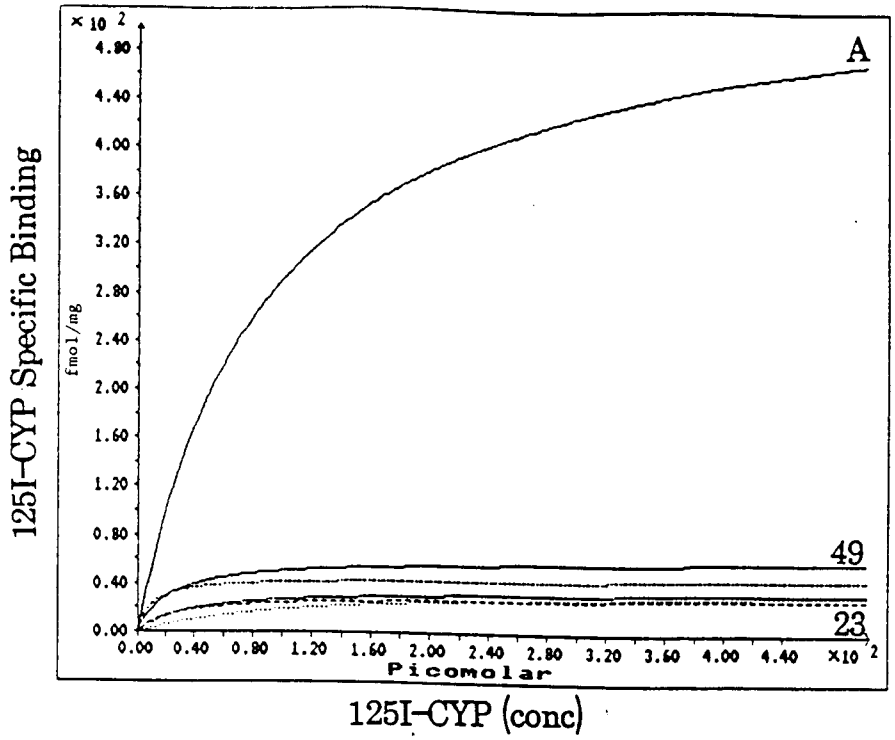


Figure 9. Saturation curves of [^{125}I]-CYP binding to adult (A) and foetal guinea pig lung membrane beta-2 adrenergic receptors from 23 to 49 days gestation. Foetal lung saturation curves shown (from below upwards) were performed at 23 days (·····) 42 days (-·-·-) 47 days (——) 46 days (-·····) and 49 days (—)

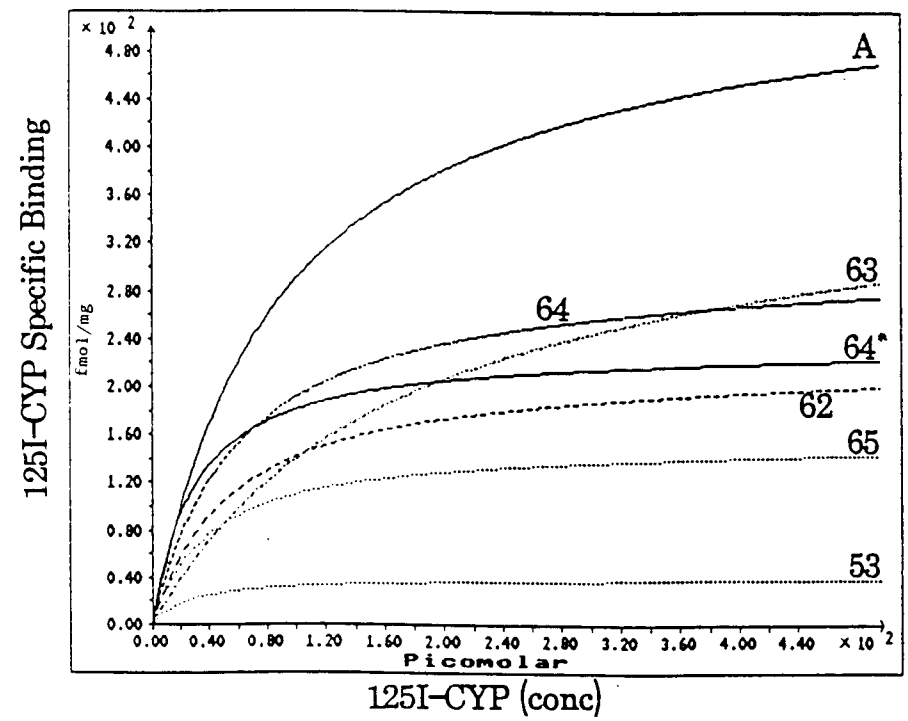


Figure 10. Saturation curves of [^{125}I]-CYP binding to Adult (A) and pooled foetal guinea pig lung membranes at 53 days (·····), 62 days (-·-·-), 63 days (-·-·-), 64 days (——), 64* days (newborn)(——) and 65 days gestation.

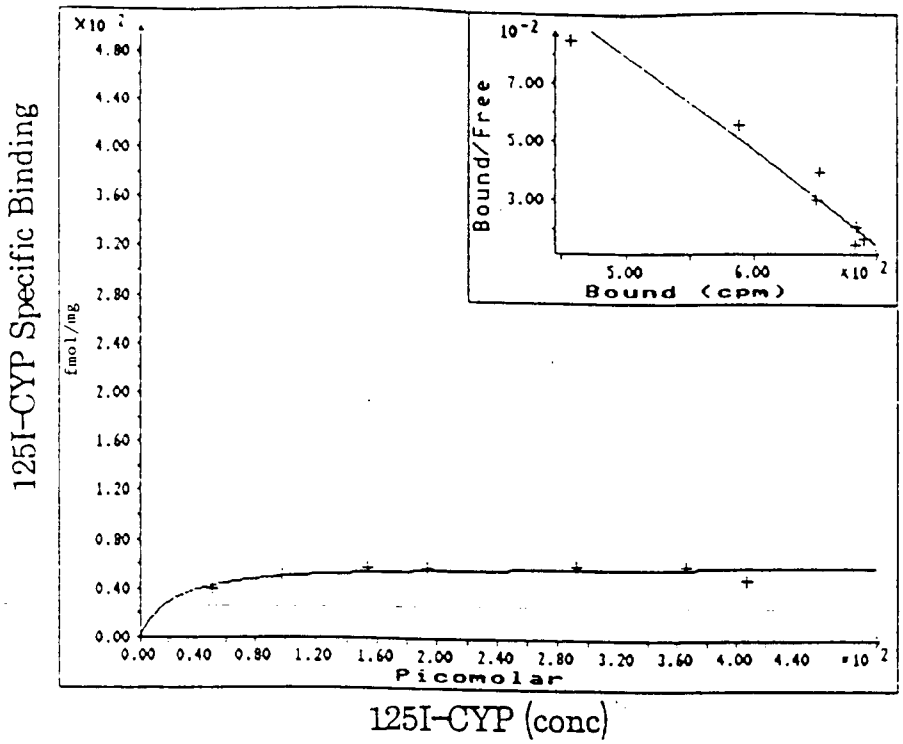


Figure 11. Saturation curve and Scatchard plot (insert) of [^{125}I]-CYP binding to pooled foetal lung membranes at 49 days gestation (Bmax = 64 fmol/mg; Kd = 27 pM)

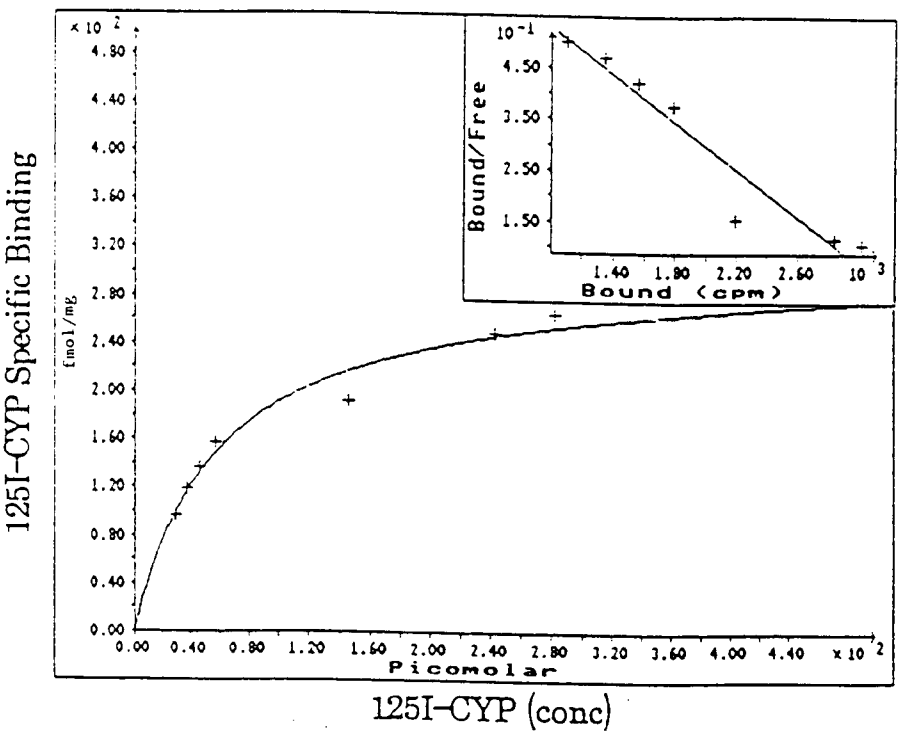


Figure 12. Saturation curve and Scatchard plot of [^{125}I]-CYP binding to pooled guinea pig lung membranes at 64 days gestation (Bmax= 260 fmol/mg; Kd = 32 pM)

Having defined the 53-62 day gestational period as the critical period for beta receptor induction in the guinea pig, it will be important to re-examine this period in detail with the view to trying to identify the serum/hormonal/genetic factors which induce receptor expression at this gestational point and in particular to study mRNA induction in the foetal lung. In view of the expense and complexity of repeating this study, I postponed further studies on the guinea pig foetal lung beta-2 receptor expression until I had acquired the genetic probes to study beta-2 receptor on RNA induction. RNA studies however proved difficult and are discussed in Chapter 4.

2.3.4 Adenylate Cyclase Activity in the guinea pig foetal lung.

Adenylate cyclase activity was studied at 9 points during the 68 day gestation period by the method adapted from Salomon et al (1979) and Whitsett et al (1981) in 2.2.12, using fresh homogenates of pooled guinea pig foetal lung. Guinea pigs were killed by asphyxiation in CO_2 and foetal and maternal lungs were removed immediately and placed in ice cold buffer. Adenylate cyclase was assayed in both foetal and maternal lungs in the same assay, the maternal lung acting as a "positive control" and reference for each experiment (Table 6). With two of the litters the sodium fluoride and isoproterenol stimulation of maternal lungs was well below the other maternal control values and hence the data obtained on the corresponding foetal lungs was discarded.

Basal adenylate cyclase activity was consistently found to be markedly elevated in the early gestation period (Fig. 13). At 23 days mean

Table 6

GESTATIONAL STUDY OF ONTOGENY OF ADENYLATE CYCLASE ACTIVATION IN THE FOETAL GUINEA PIG LUNG

Code No.	Gestation (days)	Size of litter	Basal								10mM NaF Stimulation					
			Pooled foetal values				Maternal values				Pooled foetal			Maternal		
			^{14}C cpm (mean)	Protein assayed μg	cpm/mg $x10^{+3}$	cAMP pMoles/ mg/ 15min	^{14}C cpm (mean)	Protein assayed μg	cpm/mg $x10^{+3}$	cAMP pMoles/ mg/ 15min	^{14}C cpm (mean)	cpm/mg $x10^{+3}$	% stimulation	^{14}C cpm (mean)	cpm/mg $x10^{+3}$	% Stimulation
F2/19-3	23	3	5469 6693 (6081)	47	129	217.9	504 947 (725)	77	9.4	15.8	6890 7163 (7026)	149.7	27	3042 3119 (3130)	40.6	331
F8/3-4	46	3	3855 3697 (3775)	51	74.3	125.5	720 826 (773)	65	11.8	19.9	4238 4173 (4205)	82.4	11	4704 3398 (4051)	62.3	424
F5/1-4	47	5	3173 2580 (2876)	38	75	127.7	616 612 (649)	67	9.6	16.3	3632 3559 (3595)	94.6	25	2017 2323 (2197)	32.8	241
F9/3-4	49	5	4867 4013 (4841)	51	94	158.7	ND	-	-	-	5370 4673 (5021)	98.4	3	ND	-	-
F14/19-4	62	3	2412 2702 (2557)	63	35.8	60.4	ND	-	-	-	3979 3795 (3887)	61.6	72	ND	-	-
F15/19-4	65	3	2260 2090 (2175)	63	34.5	58.2	698 768 (733)	171	4.2	7.05	2649 3171 (2910)	46.1	33	3496	20.4	376
F4/19-3	68	4	1609 1866 (1866)	59	31.6	53.3	518	53	9.7	16.3	4743	80.3	154	2042 1737 (1889)	35.6	267

Table 6 (Cont.)

ISOPROTERENOL + GTP STIMULATION (GUINEA PIG FOETAL LUNG)

Code No.	Gestation (days)	Basal pMoles/ mg/15min	Isoproterenol (10^{-6}M) + GTP (10^{-6}M) (Pooled foetal)*** cpm** (mean)	cpm/mg	pMoles/mg 15min	% Stimulation*
F2/19-3	23	217.9	6251 6256 (6257)	133×10^3	224.6	3.1%
F8/3-4	46	145.1	3436 4089 (3672)	72×10^3	140.6	0
F5/1-4	47	127.7	3063 3259 (3147)	82.8×10^3	159.2	9.5%
F9/3-4	49	158.7	4633 5398 (5015)	98.3×10^3	192.0	9.9%
F14/19-4	62	60.4	4104 3905 (4005)	63.5×10^3	107.2	77.4%
F15/19-4	65	58.2	2970 3545 (3280)	51.0×10^3	99.6	71.1%
F4/19-3	68	53.3	3306 3206 (3256)	55.1×10^3	93.2	74.8%

* % Stimulation = $100 \times \frac{\text{Stimulation - Basal}}{\text{Basal}}$

** $^{14}C(U)$ Specific activity 593mCi/mMole; 592 cpm = 1 pMole; B-counter 45% Efficiency (Calc.)

*** Iso (10^{-6}M) + GTP (10^{-6}M) stimulation or maternal lungs = 77% and 94%

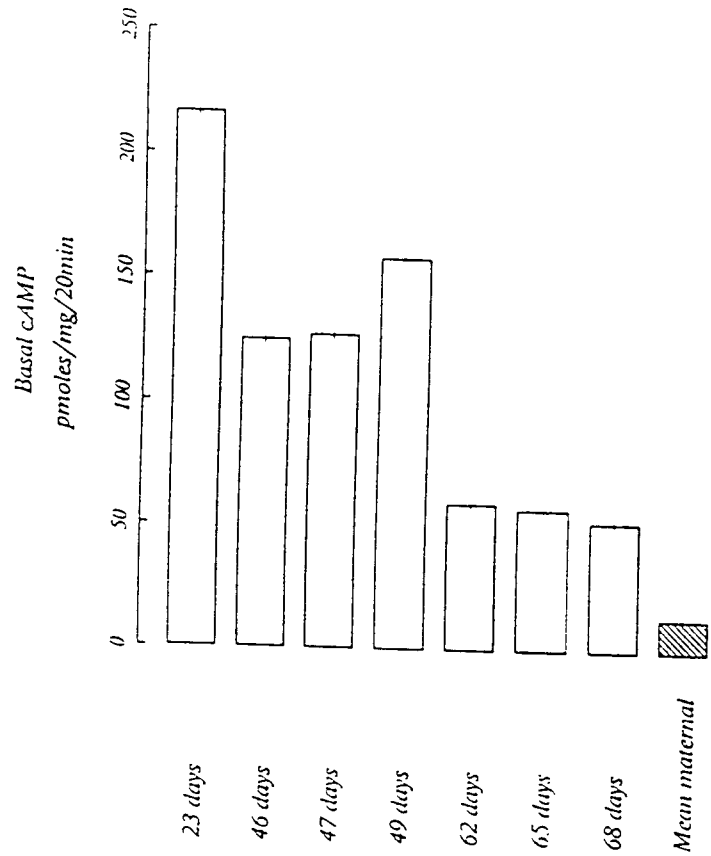


Figure 13. Mean basal adenylate cyclase activity in foetal guinea pig lungs measured by production of $^{14}C(U)$ cAMP (pMoles/mg/15min) at 23, 46, 47, 49, 62, 65 and 68 days gestation. Values for five maternal lungs, designated 1-5 are shown on the right. (Actual values are shown on Table 6).

basal cyclic AMP levels were 217 pmol/mg/15 min, but fell progressively with increasing gestation. Levels of basal adenylate cyclase activity observed in the maternal guinea pig lung homogenates were much lower (Mean cAMP = 15.1 pmol/mg/15 min, range 7.05-19.9 pmols/mg/15min), than in the foetal lung homogenates.

Isoprenaline (10^{-6}M) stimulatable adenylate cyclase activity in foetal lungs was poor up to 49 days gestation. However a sharp increase in 10^{-6}M isoprenaline stimulated adenylate cyclase activity was found at 62 days which was equal to adult levels. (Fig.14).

Sodium fluoride stimulatable adenylate cyclase activity was also low up to 49 weeks gestation and found to have increased by 62 days. (Fig. 15).

Superimposition of the data obtained from the ligand binding assays (2.2.3) on the data obtained with functional assays of adenylate cyclase activation showed that the gestational increase in both sodium fluoride and isoproterenol stimulated adenylate cyclase activity accompanied the increase in beta adrenergic receptor expression, that significant increases occurred between 49 days and 62 days gestation and that the beta adrenergic receptors induced were functional.

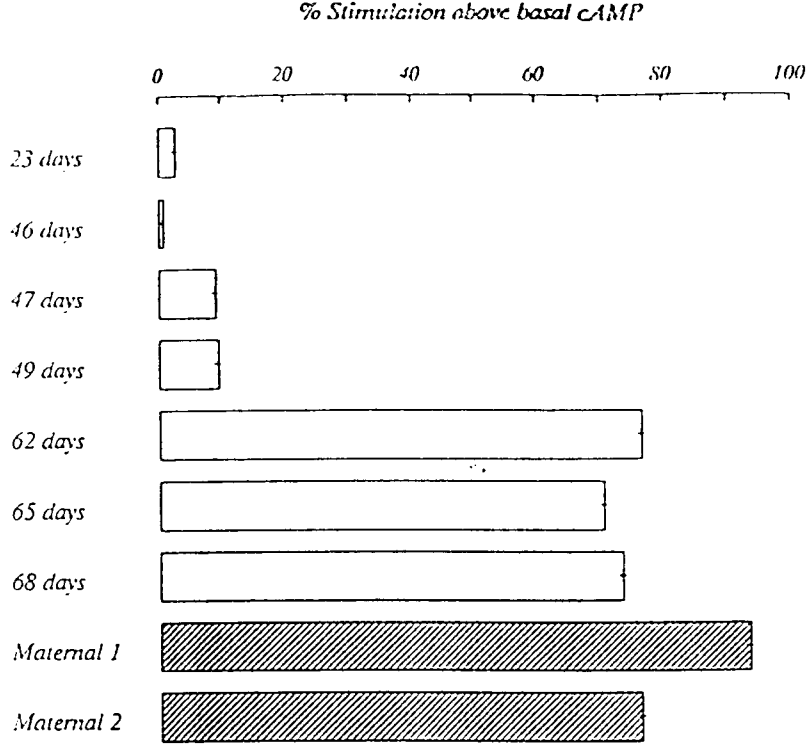


Figure 14. Mean 10^{-6}M isoproterenol stimulation of cAMP production (expressed as % stimulation above basal levels, see Fig. 13) in homogenates of guinea pig foetal lungs at 23, 46, 47, 49, 62, 65 and 68 days gestations. (Two maternal values designated M1 and M2 shown on the right). (Data derived from Table 6).

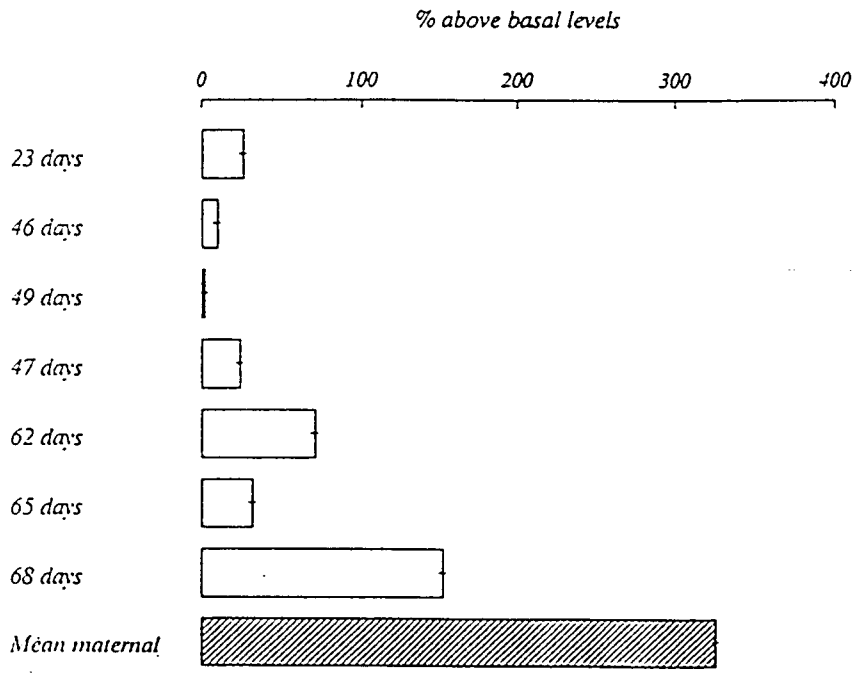


Figure 15. Mean 10mM sodium fluoride stimulation of cAMP production (expressed as a % stimulation above basal levels, see Fig. 13) in homogenates of guinea pig foetal lungs at 23, 46, 47, 49, 62, 65 and 68 days gestation. (5 maternal values designated 1-5 are shown on the right). (Data derived from

2.4 Identification of beta adrenergic receptors in mid trimester human foetal lungs.

Studies of the expression of beta receptors and adenylate cyclase activity in human foetal lungs have not been previously published. In order to investigate the functional integrity of beta adrenergic receptors in the developing human mid trimester foetal lung, I obtained lungs from fresh abortuses from pregnant women from 13 weeks to 28 weeks gestation. The foetuses were gestationally aged using ultra sound head circumference charts (Groote Schuur Hospital). Lungs were removed and stored in liquid nitrogen for assay of both beta receptors and adenylate cyclase activity. Unfortunately binding data from spontaneous abortions was not found to be reliable.

Lungs obtained from 4 therapeutic abortuses were also studied, since it was possible to remove the lungs within minutes of death. Data demonstrating adenylate cyclase activity in foetal lungs obtained from a therapeutic abortions (performed for anencephaly) at 13, 15 and 18 weeks is shown in Table 7.

Table 7.

Adenylate cyclase activity in mid trimester human foetal lungs

Gestation (weeks)	Mean Lung weight (g)	% Stimulation sodium fluoride (10mM)	% Stimulation isoproterenol (10^{-6}M)
13	0.8	80	14
13	1.1	100	16
15	1.9	65	44
18	2.8	75	52

Although the numbers of these studies are small the results do, in fact, indicate that certain adrenergic receptors in the crude human foetal lung homogenates are in fact functional, early in mid trimester. The significance of these findings would have to be tested in an extended gestational study. Of greater importance would be to define the cells in which beta-2 receptors are induced. However, the ethical and practical problems which are involved in obtaining fresh viable human foetal lungs as they approach term preclude further studies of this nature until non-invasive methods are developed to measure beta-2 receptors *in vivo*.

2.5 Summary and Discussion

The experiments described in this chapter were conducted to set up reliable techniques for the measurement of beta adrenergic receptors as a foundation for the serum studies I had planned and have described in Chapter 3. Although most of the techniques were based on previously published methods, by looking critically at the assays, I have found that certain modifications were necessary to improve their reliability. Modifications which I have introduced and major findings of the experiments in this chapter may be summarised as follows:-

1. During isolation of the cell membranes from guinea pig lungs I have added the protease inhibitors 1 mM NPGB and 1mM EGTA to inhibit inherent protease activity. Protease inhibitors were not used in the membrane preparations of Strauss (1979) or Venter (1980).
2. I have used [^{125}I]-CYP rather than [^{125}I]-HYP as the beta-2 receptor ligand in view of its greater specificity.
3. Since a wide range of maximal [^{125}I]-CYP binding was obtained for different adult guinea pig lungs (range 180-670 fmol/mg) it is important to use a pool of guinea pig lungs to obtain a "standardised" membrane population if assays are to be conducted over a period of days or weeks.
4. Saturation curves and Hill plots confirm that the [^{125}I]-CYP binding to guinea pig lung membranes obeys Michaelis Menten

kinetics and that one molecule of ligand binds one molecule of receptor.

5. Competition curves using ICl 118, 551 confirmed that the guinea pig membrane preparations contain a mixed population of beta receptors, previously shown to be predominantly beta-2 (Engel 1981).

6. Although glycerol sucrose cryopreservation (Crawford 1984) can reliably be used to store guinea pig lungs for receptor binding assays for up to two months, liquid N_2 is superior for long term storage.

7. The method of Penefsky (1977) for separating bound from free ligand was not suitable for beta receptor membrane binding assays.

8. A fairly wide range of Mg^{++} (1-15mM) is permissable in the [^{125}I]-CYP ligand binding assay.

9. Photo-affinity labelling of membranes using [^{125}I]-CYP-diazirine showed L(-) propranalol displacable binding in the 68-90000 daltons molecular weight region.

10. In the functional adenylate cyclase assays using fresh crude guinea pig lung homogenates, comparable % stimulation by sodium fluoride or isoproterenol was obtained when [α^{32}P]-ATP or [U-^{14}C] ATP was used as a substrate.

11. In the guinea pig foetal lung an increase in beta adrenergic receptor expression is found after 53 days which is accompanied by a fall in basal adenylate cyclase activity and an increase in isoproterenol and sodium fluoride induced stimulation of cyclic AMP.

13. Increasing isoproterenol inducible adenylate cyclase activity is present in the human fetal lung as early as 15 weeks gestation.

Studies of the expression of beta adrenergic receptors and the ontogenic changes in cell or organ sensitivity of beta receptors to beta receptor agonists provide temporal clues to the presence of inducing regulatory factors, particularly when defined periods of increased receptor expression are documented. By studying a well defined developmental stage of increased receptor expression, inducing factors are more readily identified. In the case of the rabbit, the foetal lung beta adrenergic receptor expression has been shown to increase towards term (Giannopoulos 1980), and is associated with increased surfactant production. This increase is believed to be stimulated by endogenous glucocorticoids (Cheng et al. 1980). In the rat lung, beta adrenergic receptor expression increases 11-fold from day 18 of gestation to day 28 of post natal life (46 ± 7 to 491 ± 69 femtomole mg^{-1} protein) and this increase in beta adrenergic receptor number is accompanied by an increased percentage stimulation by isoproterenol and sodium fluoride (Whitsett et al. 1981).

The critical period of induction of guinea pig lung membrane receptors in foetal life has not been previously reported. The results of the studies which I have performed on the developing guinea pig lung are similar to the rat studies, and beta receptor expression was found to be quiescent for 80% of the gestation period (53 days out of the 68 day

gestation period in this study). This indicates that increased beta adrenergic receptor expression is not necessary for early lung growth and development (up to 25% of adult lung weight). Increased receptor expression occurred after 53 days but the affinity of the beta receptor for the ligand [^{125}I]-CYP did not vary significantly during development as in the studies with rabbit lung by Giannopoulos (1980). Since lung weights also increased more rapidly after 53 days gestation, it is possible that increased receptor expression could simply reflect increased cellular mass and that the growth and differentiation factors influencing cell mass indirectly induce beta receptor expression. A moderate variability in the wet lung weights within the same litter was observed. Foetal lung weights were also quite variable when lungs of similar gestational ages were compared and increased lung weight was not always associated with increased receptor expression. It would have been more accurate to measure dry lung weights, but this was not possible, since in this study the fresh lungs were used for adenylate cyclase assays after they were weighed.

Of great interest was the finding of a high basal adenylate cyclase activity early in gestation (at 23 days) which fell progressively during foetal development, but at term did not reach the much lower levels encountered in adult guinea pig lung homogenates. The reasons for such high basal adenylate cyclase activity early in gestation are not known. High basal adenylate cyclase activity has also been reported in the immature rabbit lung by Barrett et al. (1974), and may represent an "unregulated" or higher activity of an enzyme which couples with receptors other than the beta adrenergic receptor.

The developmental data obtained in these studies does not differentiate between the induction of beta-1 and beta-2 receptors in the lung membranes. The differential induction of beta receptors in different cell types in the lung must be considered, since it is well known that beta-1 and beta-2 receptors may be independently regulated (Table 3 and Table 4).

Following the studies of cortisol enhanced isoproterenol induced surfactant secretion in the A549 pulmonary adenocarcinoma cell line (Smith 1977), Dobbs and Mason in 1979, in a study of pulmonary alveolar type II pneumocytes isolated from rats, found that treatment with beta adrenergic agonists caused an increase in the release of [^{14}C] desaturated phosphatidylcholine from type II cells in culture. Maniscalco and Shapiro (1983) have, using foetal rat lung explants, shown that glucocorticoids have a direct effect on foetal lung to increase beta adrenergic receptor density and that new protein synthesis was required for this effect. It is thus likely, on evidence published to date that the gestational surge in beta receptor expression, at term, is due to glucocorticoids.

It has been suggested that while steroids only cause a modest increase in beta receptor density in the normal adult lung. The effect is much more pronounced when the density of beta receptors is reduced due to diseases such as bronchial asthma (Salonen 1984) and under conditions associated with a lower level of cellular differentiation, such as the foetal state. This suggests that pulmonary beta receptors are subject to regulation by factors that

affect cellular growth and differentiation. It is tempting to postulate that disorders in the release or control of such factors could also contribute to pathology of the beta-receptors in certain respiratory diseases. Recently the regulation of beta-2 receptors in hamster smooth muscle cells has been studied at the level of mRNA (Collins et al 1988).

Reasons for the relative resistance of lung beta receptors to induction by steroids in normal adults, compared with term foetuses, are unknown. Stern and Kunos (1988) have shown that lymphocytes and macrophages may be involved in a paracrine regulation of beta receptors in surrounding lung cells. Furthermore, the importance of appropriate beta-2 receptor expression in neuronal development is emphasised by the demonstration by Schwartz (1988) that nerve growth factor mRNA is also stimulated by exposure of C6 glioma cells to isoproterenol, suggesting that a complex relationship may exist between functional beta-2 receptor expression and neuronal development.

Recently Skinner et al. (1989) have also shown that there is a differential selectivity between fibroblasts and type II epithelial cells in their adenylate cyclase responses to beta agonists and prostaglandin E2 in the foetal rat lung.

Barnes et al. (1983) and Carstairs et al. (1985) using autoradiographic localization have demonstrated that most of the beta receptors in the mature lung are in the conducting airways and in the alveolar epithelium and that reduction in terminally distributed beta receptors

occurred in the ovalbumin guinea pig asthma models. Thus, identification of the inducing and regulatory factors of the conducting airways and terminally distributed alveolar epithelial receptors may be of importance in understanding mechanisms underlying the pathophysiology of allergic asthma. It is as yet not clear whether these changes are a primary cause of the asthma, or are secondary to the release of immunological and biochemical activation products from the cellular infiltrates occurring in the airways in allergic asthma.

Clearly ontogenic changes in cell sensitivity depend not only on changes in beta adrenergic receptor sites expressed,but will depend upon the catecholamine concentration at the site, the affinity of the receptor for agonist, the state of the membranes, G proteins and post receptor transduction mechanisms and cell differentiation.

The ontogenic model of beta-2 receptor expression in the guinea pig is an important physiological developmental model which should be explored. There are clearly not only factors which facilitate steroid induction of beta-2 receptors at the end of gestation, but others which maintain the beta-2 receptor expression at the higher level and induce a relative resistance to further induction by steroids in mature animals. The optimal number of receptors/mg membrane protein for different cells has not yet been studied. From the developmental studies I have performed in the guinea pig it is apparent that adequate and normal pulmonary function is possible in new born guinea pigs even although they have a complement of beta-2 receptors per mg lung membrane which is only half the complement found in adult guinea pigs.

Furthermore, the wide variation in the number of beta-2 receptors per mg lung membrane which I found expressed in different adult guinea pigs suggests that a fairly marked overall reduction in beta-2 receptor per mg lung membrane is permissable in an animal which is highly susceptible to the development of asthma. Until the relationship between numerical expression and functional capacity can be determined with certainty, data attributing only modest reductions to the primary development of asthma should be interpreted with caution. I have studied the effect of receptor number on adenylate cyclase activation by isoproterenol using mouse L cells which I transfected with the human beta-2 adrenergic receptor gene, in Chapter 6.

It is likely and highly desirable that in the normal mature animal a certain receptor reserve or excess should be present to compensate for factors causing partial beta receptor blockade or down regulation particularly when adequate function of a receptor such as the beta-2 adrenergic receptor is critical for life. Unless "blocking factors" - previously described by Venter et al (1980) and Blecher et al (1984) reduce the receptors below the critical functional level of expression for normal, or adequate adenylate cyclase responses to catecholamine, their relevance to clinical disease is likely to be small.

CHAPTER 3

Inhibition of [^{125}I]CYP binding to guinea pig lung membrane beta-adrenergic receptors by human serum.

3.1 Introduction

Unimpaired binding of circulating catecholamines to beta-adrenergic receptors to airway smooth muscle is critical for maintenance of the balance between the cholinergic and the adrenergic nervous systems and hence the maintenance of normal airway tone in asthmatics (Griew and Pierson 1971, Ind et al. 1985, Warren et al. 1984). Blocking of lung beta adrenoceptors by high affinity antagonists e.g propranalol results in bronchospasm in asthmatic subjects. Circadian falls in catecholamine levels correlate well with nocturnal wheezing (Barnes 1980a) and partial defects in adrenaline release in asthmatics during exercise (Warren et al. 1982), acute asthma (Ind et al. 1985) and isocapneic hyperventilation (Barnes et al. 1981) are well described.

The possibility that serum factors, in particular, may also play a modulatory effect on circulating catecholamine binding to beta adrenergic receptors, was raised by the studies of Fraser et al (1981) who found that dilutions of serum from asthmatics and certain non asthmatic individuals could indeed inhibit [^{125}I]HYP binding to canine and calf lung membrane beta adrenergic receptors.

Although these studies raised very interesting possibilities in relation to asthma when they were initially published, there has only been one published study (Blecher et al. 1984) to support the initial data. The pathogenetic significance of the inhibitory effects on antagonist binding has not been resolved to date, since detailed *in vitro* studies investigating the nature of this phenomenon have been undertaken. In some quarters the original studies of Fraser and Venter have even been regarded with a degree of sceptism, and recently, (Kokubu et al. 1989) using canine lung membranes reported that they could find no evidence for autoantibodies to the beta adrenergic receptor in asthmatics in Japan. By contrast the evidence for autoantibodies to beta-1 adrenergic receptors is mounting. Limas (1989a) has found that sera from certain patients with idiopathic dilated cardiomyopathy inhibit [^{3}H] alprenalol binding *in vitro* and that following cardiac transplantation, the inhibitory effect of the sera decreased. Evidence for autoantibodies to beta-1 adrenergic receptors in human cardiomyopathy has also been reported by Sterin Borda et al. (1984) and Borda et al. (1984a).

An investigation into possible inhibitory effect of serum on ligand binding to beta-2 receptors is important in asthmatics. Beta-2 receptors on smooth muscle in the lung are not normally bathed in serum. If, however, during bacterial inflammation, for example, in chronic bronchitis, or as part of the allergic inflammatory response following mast cell degranulation, or viral infection, beta-2 adrenergic receptors are exposed to serum, endogenous catecholamine binding to beta adrenergic receptors in smooth muscle, and other cells

in the airways may be blocked by these serum factors. The use of high affinity beta-2 agonists may be necessary to overcome any blocking effect of serum factors to restore normal airway calibre, particularly in those asthmatic individuals whose airways are critically dependent on an intact adrenergic drive.

It is, important to identify the serum factor(s) mediating receptor blocking, their regulation and the role they play in flare-up of asthma, since these factors may influence the effectiveness of beta-2 stimulators in the therapy of asthma.

A pilot study was conducted (3.2.1), firstly to investigate whether [^{125}I]-CYP binding was inhibited by serum in a reproducible fashion using the guinea pig lung membranes and secondly, to determine whether there was in fact any statistical evidence that the inhibitory effects found with asthmatic sera were any different to those found with normal control sera. Based on the results of the preliminary study, I then conducted further experiments in which the inhibitory serum effects from groups of asthmatics, in different clinical categories of severity, are compared (3.2.2).

These experiments were extended in a specific study of the effect of purified IgG on ligand binding (3.3), and an investigation into the cross species immunogenicity of lung membranes containing beta adrenergic receptors in the New Zealand White rabbit.

3.2 Inhibition of [^{125}I]-CYP binding by human sera.

3.2.1 Preliminary Study.

(i) Sera

(a) Asthmatic sera.

10ml clotted blood was taken from 54 children with atopic asthma sequentially attending the Allergy clinics of the Red Cross Childrens Hospital, Cape Town. All children had elevated IgE levels (PRIST, Pharmacia) for age (Kjellman, 1976) and positive skin or RAST tests (Pharmacia, Uppsala) to two or more common environmental allergens. Serum was aliquotted and stored at -20°C.

(b) Non-asthmatic control sera.

10ml clotted blood was taken from 24 children who did not have a history of allergic disease or a family history of allergy. All of the sera had normal IgE levels. Sera were aliquotted and stored at -20°C. None of the controls were on any drug medication at the time of collection of the sera.

(ii) Ligand inhibition assay.

50μg of guinea pig lung membrane protein was preincubated with a 1/50, 1/250 or 1/500 dilution of serum from asthmatic or non-asthmatic control subjects, for 1 hour in 20mM sodium phosphate buffer 2mm $MgSO_4$ pH 7.6, prior to the addition of [^{125}I]-CYP at the Kd for beta receptors in the guinea pig lung membrane

preparation, (determined by Scatchard analysis to be 30pM) and 10^{-6}M L(-) propranalol (or buffer) for 30 minutes at 30°C. At the end of the incubation period, the reaction was stopped by the addition of 1.25ml cold 20mM potassium phosphate buffer, 2mM $MgSO_4$, pH 8.0 and 1 mM D-L propranolol. Samples were immediately filtered under low vacuum through Whatman GF/C glass fibre filters, the filters were then washed with 25ml 2mM $MgSO_4$ 20mM potassium phosphate buffer and sucked dry under vacuum using a 6 place Millipore filter (Millipore Incorp.) and radioactivity retained on the membranes was measured using a gamma counter (Beckman Gamma 4000). Specific binding was determined by subtracting the radioactivity retained on the Whatman GF/C filters from samples incubated in the presence of (-) propranalol, from the radioactivity retained from samples incubated in the absence of (-) propranalol.

(iii) Paediatric asthmatic sera compared with non asthmatic control sera:-

Inhibition studies were conducted using 1/50, 1/250 and 1/500 dilutions of sera. Fig. 16 shows the serial inhibition of specific [^{125}I]-CYP binding obtained with 5 typical asthmatic sera compared with a non-asthmatic control serum (lower graph). Both asthmatic and non-asthmatic demonstrated dose responsive inhibition of [^{125}I]-CYP binding to lung membrane beta receptors.

In the comparative study inhibition of [^{125}I]-CYP binding by a 1:100 dilution of serum from 54 atopic asthmatic children was

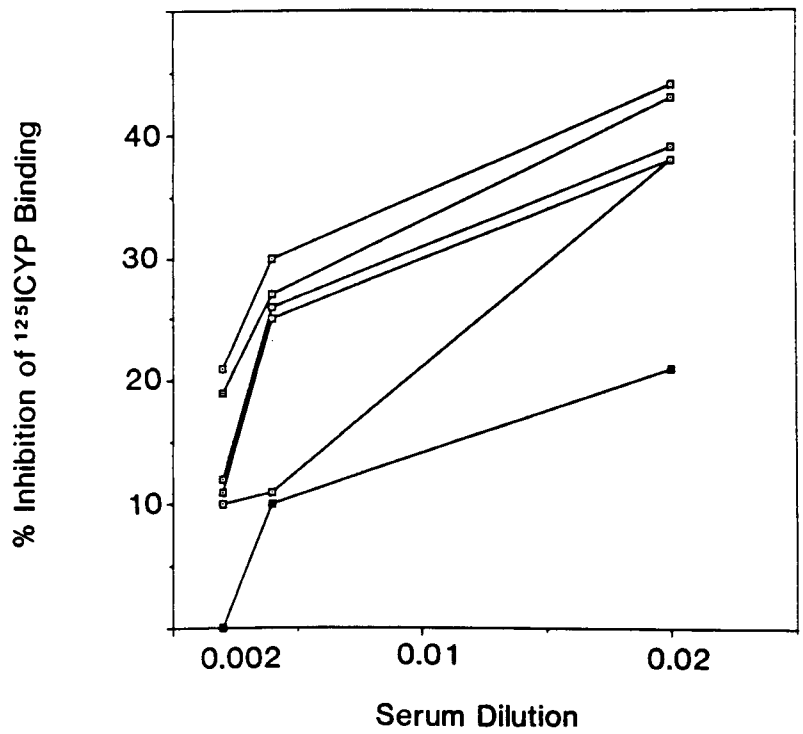


Figure 16. Serial inhibition of [^{125}I]-CYP binding to guinea pig lung membrane beta receptors using sera from 5 asthmatic children (□). Serial inhibition was also observed with non-asthmatic sera (■).

compared with the 23 non-asthmatic, non-atopic controls. % Inhibition obtained is shown in Fig.17 and summarised as follows:

	Number	Mean inhibition (SEM)	Range
Asthmatic sera	54	27.4 ± 2.97	0-78%
Control sera	23	11.08 ± 3.42	0-54%

The difference in the mean inhibition between the two groups was highly significant $p = < 0.0026$ t-test ($p=0.0018$ Kruskal-Wallis Test), but there was clearly a marked overlap between the groups.

3.2.2 Inhibition of ^{125}I-CYP binding to beta receptors by sera in different severity categories.

Since certain sera from unselected asthmatic children inhibited the binding of [^{125}I]-CYP to guinea pig lung membrane beta receptors significantly more than serum from the non-asthmatic control subjects in the preliminary study, further inhibition studies were performed in a prospective study to investigate whether inhibition was a feature of mild or severe asthma and whether significantly greater inhibition of binding was present in the sera of asthmatic patients during an acute attack of asthma.

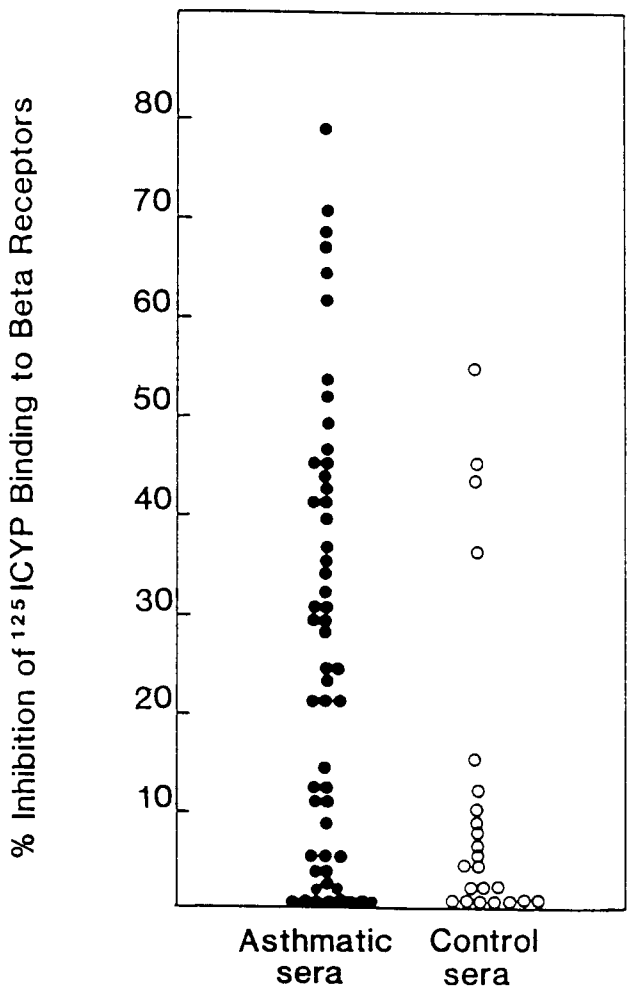


Figure 17. Inhibition of [^{125}I]-CYP binding to guinea pig lung membrane beta receptors by asthmatic sera (1:100 dil.) N=54 and control sera (N=23).

(i) Patients

Sera from 76 patients with asthma in different clinical categories were obtained. Sera were obtained from patients in each group consecutively, within a period of one month, in the order in which they presented to the Allergy clinic or asthma emergency room to avoid bias in selecting the patients.

Categories of asthmatic subjects studied as follows:

Group	No. of patients	Medication			Asthma status	Clinical category
		Prednisone	Salbutamol	Theophylline		
B	17	-	±	±	Stable	mild-moderate
C	23	+	+	+	Stable	Severe
D	21	+	+	+	AA	Severe
E	15	-	±	±	AA	mild-moderate

Group A was a control group of non asthmatics.

+ = Daily maintenance therapy

- = No medication

± = on intermittent or daily therapy

Stable = not having an acute attack at time of sampling

AA = acute attack of asthma at the time of sampling

For this study, patients were categorised as severe if they were on daily prednisone, as well as requiring both salbutamol and theophylline to control their disease.

Sera were coded, aliquotted and stored at -20°C until used in the [^{125}I]-CYP inhibition assay or for IgG purification.

A pooled guinea pig lung membrane preparation expressing 555fmol/mg protein beta receptors (i.e. 27.75fmol beta-2 receptors per assay point) was used for the ligand inhibition assays. Patient sera were studied at both 1:50 dilution and a 1:250 dilution of the serum, in 16 consecutive assays, with dilutions of each sample studied in triplicate.

To monitor reproducibility of the [^{125}I]-CYP inhibition obtained with the sera, 30 sera were re-tested in 16 assays and follow-up results were compared with initial results obtained. In general, results were acceptably reproducible in the samples re-tested.

An analysis of variance of interassay results of the degree of serum inhibition (N = 30) showed good reproducibility, with no significant difference in the results obtained in repeat assays using the Sign-Rank test ($p = 0.2131$).

Dialysis of the serum against 20mM sodium phosphate buffer pH 7.4 overnight or pre-incubation of the membranes with serum for up to 3 hrs did not make any significant difference to the degree of inhibition obtained in the [^{125}I]-CYP inhibition assay.

(ii) Results

The mean of inhibition of [^{125}I]-CYP binding obtained with 1:50 dilution of sera from 4 categories of asthma patients (B-E) is shown in the following table, and compared with the control group:

	CONTROLS	ASTHMATICS			
Category	A	B	C	D	E
Number	25	17	23	21	15
Mean % inhibition	32.92	36.64	40.65	35.22	37.2
Std deviation	7.72	18.61	17.94	6.92	11.1
Std deviation of mean	1.56	4.51	3.74	1.51	2.86
Lowest value (%)	13	21	10	20	22
Highest value (%)	49	94	83	49	66

There were no significant differences between the % inhibition exerted by the asthmatic sera within the asthma groups, or when any of the asthma groups were compared with control non-asthmatic subjects. By analysis of variance between the groups, F value = 1.09 and p = 0.367 and applying the Kruskal Wallis Test (Chi Square approximation), Chi Sq. = 4.98; p = 0.287.

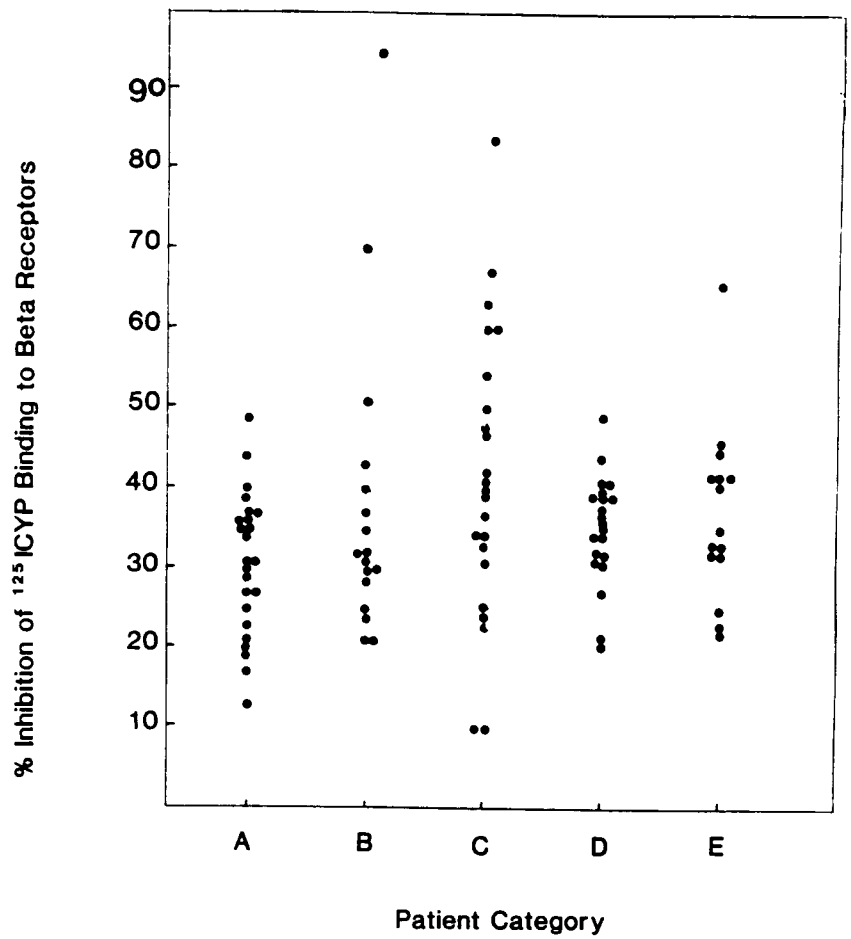


Figure 18. Inhibition of [^{125}I]-CYP binding to guinea pig lung membrane beta receptors by human sera (1:50 dil.) in 5 categories A-E (see 3.2.2).

The range of % inhibition obtained in the 5 groups studied is shown in Fig.18. 9/76 (11%) of the asthmatic sera, but none of the normal control sera produced inhibition of greater than 50%. Six out of these 9 asthmatics fell into the steroid-dependent group and on reviewing the clinical history of the one patient from category E he probably should have been on steroids. An important finding was the absence of a significantly greater degree of [^{125}I]-CYP inhibition in sera from patients during an acute attack.

Three sera were encountered, two from mild asthmatics in this study and one from a non-asthmatic, (in a follow-up study, Chapter 6) whose sera inhibited [^{125}I]-CYP binding by greater than 85% with good reproducibility of the triplicates within the assay but which was not reproducible in subsequent assays using either fresh sera or stored sera from the patients. (% inhibition in subsequent assays less than 50%). The intermittent ability of these sera to markedly inhibit [^{125}I]-CYP binding to beta receptors in membrane preparations is not explained and difficult to study further in view of the isolated nature of the phenomenon.

Analysis and comparison of the serum inhibitory capacity when studied at 1:250 dilution of serum also showed no significant differences between the groups.

Table 8

An analysis of 8 asthmatic patients whose sera inhibited [^{125}I]-CYP binding by greater than 50% at a dilution of 1:50 .

Patient	% inhibition	Treatment regime			Clinical status
		Prednisone	Theophylline	Salbutamol	
AF	54	+	+	+	Stable*
CH-C14	60	+	+	+	Stable
DL-C15	63	+	+	+	Stable
JA-C16	67	+	+	+	Stable
RY-C12	83	+	+	+	Difficult to control
ID E5	66	-	+	+	Difficult to control
B2	94	-	+	+	Stable
B3	70	-	+	+	Stable

*Stable: These patients had PEFR within predicted levels and were well controlled on their medications at the initial assessments.

A clinical follow-up of the first six patients in Table 8 over a period of five years reveals that their clinical course has been stormy, characterised by steroid dependence and repeated hospital admissions. Follow-up studies of the ligand inhibitory effect of their sera (6.6.1), showed that all of the sera on subsequent testing, using cloned human beta-2 receptors in the membrane preparation, inhibited [^{125}I]-CYP binding by less than 50%.

3.3 Inhibition of ^{125}-CYP binding to beta adrenergic receptors in lung membranes by DEAE cellulose purified immunoglobulin G

In the initial experiments by Venter et al. (1980) reporting autoantibodies to the beta adrenergic receptor it was found that selected sera which inhibited [^{125}I] Hydroxybenzylpindolol binding to canine lung membranes could also immunoprecipitate the beta adrenergic receptor. Lung membranes incubated with such sera also demonstrated increased binding of [^{125}I] protein A. These autoantibodies were thought to be of the IgG class. Blecher et al. (1984) showed that by immunodepleting IgG from asthmatic sera, using a 2.5 fold excess of goat anti-human IgG γ chain and anti-human IgA α chain, the inhibition of [^{125}I]-HYP binding to canine lung membranes could be reversed and these workers indirectly attributed the inhibitory effect to the immunoglobulin fraction in 20 of 28 high risk asthmatic sera. Direct inhibition of [^{125}I]-CYP binding to beta adrenergic receptors by purified immunoglobulin fractions has not been previously reported.

In order to verify these findings in asthmatic sera and to extend these studies to the guinea pig lung membrane, IgG fractions were purified from asthmatic sera and applied directly to the [^{125}I]-CYP inhibition assay.

Since inhibitory activity was encountered more frequently in the severe asthmatics, only severe asthmatic sera were subjected to DEAE cellulose purification. Total immunoglobulin G levels were measured in 26 non-asthmatic sera and compared with IgG levels in 19 severe asthmatics, randomly chosen from Groups C and D (3.2.2), by Nephelometry using a

Beckman Immunochemistry analyser. There was no significant difference between the IgG levels in the asthmatics and non-asthmatics (Table 9), using Wilcoxon - 2 sample test p=0.703 and Kruskal-Wallis test p=0.694.

Table 9: Comparison of the total IgG levels in 24 non-asthmatic children with 19 severe asthmatic children

	Non-asthmatic	Severe asthmatic children
N	24	19
Mean IgG(g/L)	10.05	10.50
SEM	0.355	0.84
Range (g/L)	7.0-14.5	6.00-20.0

3.3.1 IgG purification

A saturated ammonium sulphate (SAS) solution was prepared by dissolving 760g of ammonium sulphate in 1 litre of distilled water at room temperature and the pH was adjusted to 6.5 with ammonium hydroxide. Gammaglobulins were precipitated out by adding 1 volume of SAS to 2 volumes of serum, thus effecting one third saturation and adjusting the pH to 7.8 with 0.5N NaOH. After stirring for 3 hours, the suspension was centrifuged at 1400g for 30min and the precipitate was suspended in the original serum volume in normal saline. In order to remove the $(NH_4)_2SO_4$, the sample was dialysed overnight against 15mM sodium phosphate buffer pH 8.0.

Following dialysis the globulin fraction was chromatographed on DEAE cellulose which had been equilibrated with 0.02M phosphate buffer pH 8.0, allowing 1 ml of packed DEAE per 50mg semi-purified globulin. IgG concentrations prior to and after purification were measured by Nephelometry (Beckman Immunochemistry Analyser). IgG recovery from the DEAE column was monitored for each specimen using an LKB ultraviolet absorption control unit UVICORD Type 8301A.

Purity of IgG

IgG fractions prepared by ammonium sulphate precipitation and DEAE cellulose chromatography were loaded on to 11% polyacrylamide gels. PAGE

of IgG fractions stained with Coomassie Blue from 4 asthmatic patients shown in Fig. 19. Bands are detected in the 150-180 kDa, and except for small traces of albumin, other serum proteins are removed by the purification procedure above.

3.3.2 IgG inhibition assays

Inhibition of [^{125}I]-CYP binding to guinea pig lung membranes by purified human IgG was performed using the same experimental protocol as the serum inhibition assays, except that dilutions of purified IgG were used in place of dilutions of whole serum. In order to ensure that an identical concentration of IgG was employed in these assays, the corresponding IgG concentrations in each serum were was measured by Nephelometry and an identical concentration of the purified IgG was added in the [^{125}I]-CYP inhibition assay, to determine whether any component of the inhibition observed in the serum inhibition assays, could in fact be attributed to the immunoglobulin G fraction of the serum. Each experiment was performed in triplicate and the inhibition obtained compared with an equivalent dilution of whole serum.

Results of a representative inhibition experiment with purified IgG from three patients are given in Table 10. Equivalent concentrations of IgG purified from 14 patients (6 non-asthmatic and 8 asthmatic) were compared with dilutions of serum.

Using the same concentrations of IgG as in the 1/50 serum dilutions used in the serum inhibition assays, purified IgG produced comparable degrees

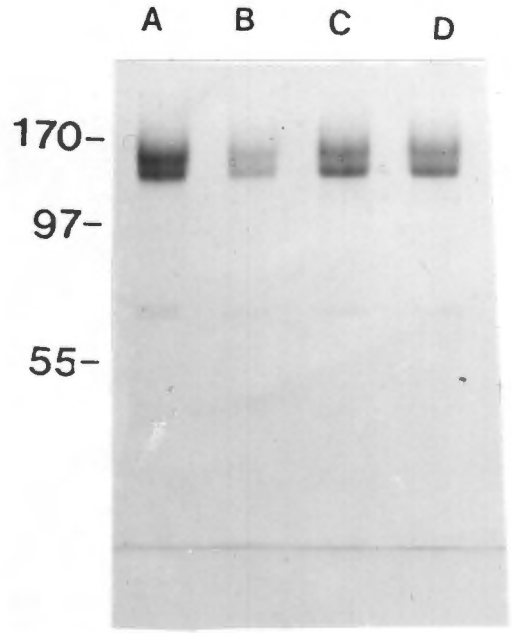


Figure 19. 11% PAGE of DEAE cellulose purified IgG fractions from 4 asthmatics (A,B,C,D) (unreduced). Molecular weight markers are shown on the left.

of inhibition to the corresponding serum in a some but not all sera. A plot of percentage [^{125}I]-CYP inhibition by 1:50 dilution of serum against percentage inhibition mediated by an equivalent IgG concentration is shown in Fig. 20. Spearman Rank correlation co-efficient = 0.075; p=0.79 confirmed that there was no direct correlation. There was also no consistent relationship between the total IgG level in a given patient and the % inhibition obtained in the inhibition assays. 13 patients were studied and % inhibition of [^{125}I]-CYP binding plotted against μg IgG/500μl in the assay (Fig. 21). Spearman Rank correlation co-efficient = 0.0248; p = 0.935.

Table 10.

Serum Code	Dilution or IgG equivalent	[^{125}I]-CYP bound cpm (mean of triplicate)	% inhibition of control binding	IgG in assay μg/500μl
No serum (control)	-	12393	-	
Falken serum	1/50	6378	50	79.5
Falken IgG	1/50	5785	54	79.5
Falken IgG	1/250	11080	18	
Falken IgG	1/500	11393	10	
Christian serum	1/50	5028	60	59.7
Christian IgG	1/50	9129	28	59.7
Christian IgG	1/250	11384	10	
Christian IgG	1/500	11735	6	
Non asthmatic 42 serum	1/50	9406	25	78.7
Non asthmatic IgG	1/50	9783	22	78.7
Non asthmatic IgG	1/250	11900	6	
Non asthmatic IgG	1/500	12527	0	
No serum(control)		12643	-	

Representative examples of the 14 patients studied are illustrated in Table 10. In the case of Falken and the non-asthmatic control, as

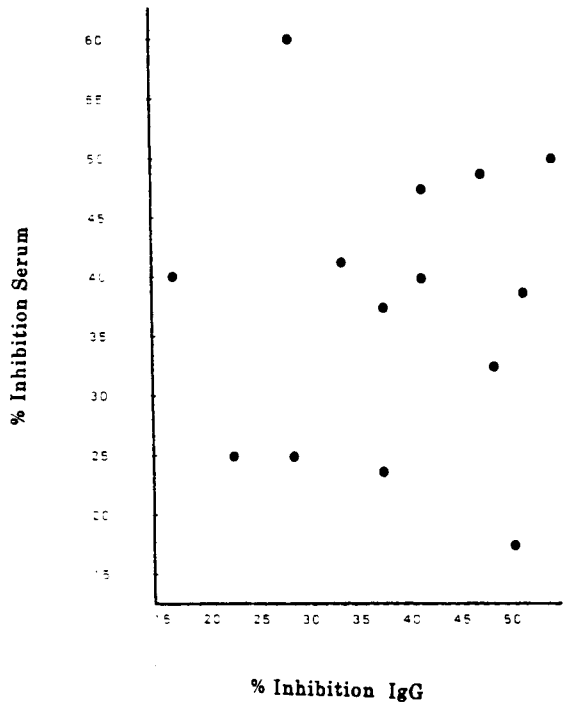


Figure 20. Plot of % inhibition of [^{125}I]-CYP binding to guinea pig lung membranes by serum (y axis) against inhibition of [^{125}I]-CYP binding by purified IgG (x axis) N=14.

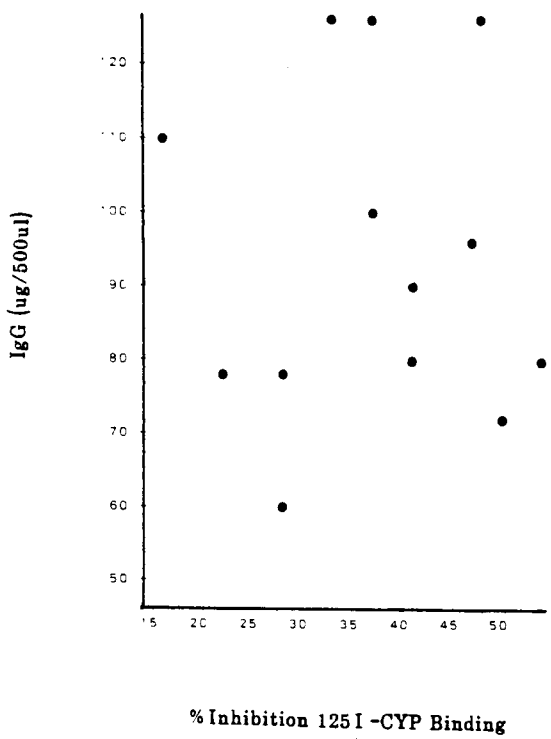


Figure 21. Plot of % inhibition of [^{125}I]-CYP binding to guinea pig lung membranes as a function of amount of IgG (μg/500μl) in the inhibition assay.

much inhibition was obtained with purified IgG as was obtained with whole serum containing an equal amount of IgG. In all instances a dose responsive inhibition mediated by IgG could be demonstrated.

In the case of Christians, a much greater inhibition (60%) was observed by a dilution of whole serum than an equivalent amount of the purified IgG alone suggesting that [^{125}I]-CYP inhibitory factors other than IgG may be present in the sera of some patients.

These experiments demonstrated that DEAE purified IgG functions from asthmatic and non-asthmatic patients can influence the binding of [^{125}I]-CYP to beta receptors in the guinea pig lung membrane, and that in most instances, the degree of inhibition approximates the degree of inhibition mediated by the equivalent dilution of whole serum. The degree of inhibition is not a function of the total IgG concentration in each patient.

As a result of the foregoing observations the following questions are raised and have been addressed in further studies reported in this thesis:

1. Is the inhibition of beta receptor [^{125}I]-CYP binding by immunoglobulin due to specific competition for the ligand binding site, or is the inhibition possibly due to steric obstruction? (6.6.2)
2. Do immunoglobulins interact with cell membranes in a non-specific way, do they bind to Fc IgG receptors or do they alter

the expression or orientation of membrane receptors so that their ability to bind ligands is impaired? (3.5)(6.4.7)

3. What is the nature of the ligand binding domain of the beta-2 adrenergic receptor? (4.3)

4. Does the degree of inhibition of [^{125}I]-CYP binding vary with the level of beta-2 receptor expression in the cell membranes? (6.6.1)

5. Does the inhibition of [^{125}I]-CYP binding by serum have functional significance with respect to adenylate cyclase activation and cyclic AMP production?

While answers to some of these questions could be partially answered by further studies using the guinea pig lung membrane preparation, it was clear to me that a more precise molecular understanding of the ligand binding site was required before specific interactions between immunoglobulins and the beta-2 receptor could be studied intelligently. The studies conducted in this chapter were performed before the beta-2 receptor gene was cloned and sequenced. In the following sections (3.3.3 - 3.5) I have described the additional exploratory experiments I performed on the guinea pig lung membranes. I have described my further investigations of this phenomenon using cloned human beta-2 receptors in Chapter 6.6.

3.3.3 Adherence of IgG to lung membranes.

In order to demonstrate that immunoglobulins do in fact associate with lung membranes either by low affinity specific binding or by other

non-specific interactions (e.g. hydrophobic, van der Waals) a [^{125}I]-Protein A assay was employed.

Briefly, 50μl of a 1/100 dilution of serum from 4 patients was incubated in triplicate with 50μl (100μg) guinea pig lung membranes (final dilution 1/200) for 60 min at room temperature in Beckman airfuge cellulose propionate 5 x 20mm tubes. After incubation membranes were washed twice with 100μl 0.1% ovalbumin in 100mM sodium phosphate buffer, pH 7.4. with 150 000cpm [^{125}I] Protein A [^{125}I]-labelled with Bolton and Hunter reagent, 30mCi/mg, Amersham 1M.112) in 100μl sodium phosphate buffer. The membrane preparation was then incubated for 30 min. at room temperature. Membranes were washed twice with 0.1% ovalbumin using the airfuge and the pellets (in the tubes) counted in a Packard model 3003 gamma counter. Controls, without serum, or without receptor were included in the assay.

Results for the Protein A assay are shown in Table 11.

Table 11

	Control no membrane no serum	Control membrane	Patient I	Patient II	Patient III	Patient IV
Mean cpm	6788	21718	23641	26174	62739	45528
% increase in [^{125}I] Protein A above binding to control receptor	-	-	10.2%	20.5%	299%	209%

Table 11 suggests that immunoglobulin G maybe binding to the guinea pig lung membranes upon incubation, as indicated by an increase in [^{125}I] Protein A binding. An increase of up to 299% of binding was obtained in the presence of a 1:200 dilution of serum from patient III. However, it was found that some of the apparent increase in binding was due to IgG sticking to the cellulose propionate tubes. The extent of this was demonstrated in an experiment in which 50μl of a 1/100 dilution of patient III serum was incubated in the cellulose propionate tubes, in the presence or absence of 0.5% Tween. Mean counts obtained when [^{125}I] protein A (100 000cpm) was subsequently added and washed were 4109 cpm in the presence of 0.5% Tween and 31801 cpm in the absence of Tween.

Coating of the airfuge tubes with 10% foetal calf serum did not reduce non-specific adherence of IgG to the tubes. The [^{125}I] protein A assay was thus repeated on sera III and IV in the presence of 0.5% Tween and results obtained when 100 000cpm [^{125}I] Protein A was added were as follows:

	Mean cpm
Control no membranes no serum	1468
Control membranes no serum	3786
Patient IV serum no membranes	2141
Patient IV with membrane protein	6780
Patient III serum no membrane	7308
Patient III serum with membrane protein	13766

Non-specific binding to the tubes was thus reduced and only a marginal but a consistent increase in the Protein A binding was observed when 1/100 serum dilutions from patient's III and IV were incubated with

the guinea pig lung membranes in the presence of 0.5% Tween. Patient III serum still demonstrated significant binding to the tubes in the presence of 0.5% Tween. These experiments demonstrated that the [^{125}I] Protein A assay (Fraser et al. 1981) has distinct limitations, and direct studies demonstrating immunoglobulin association with purified beta receptors in ELISA or Western blot are necessary.

3.3.4 The effect of varying the incubation period for membrane interaction.

The following experiment was performed to determine whether preincubation of the membrane receptor with IgG would in fact increase the inhibition of binding of [^{125}I]-CYP to membranes observed. For this study lung membranes preincubated with a fixed concentration of IgG for 10, 20, 40 and 60 min prior to the addition of [^{125}I]-CYP were compared with membranes to which IgG and [^{125}I]-CYP were added simultaneously. The effect of pre-incubation of membranes is shown in as follows:

Values shown, cpm and % inhibition are the means of triplicates.

	[^{125}I]-CYP cpm bound	% inhibition
No preincubation		
without IgG	11748	
with IgG	6270	47
Preincubation		
10 min	6745	43
20 min	5779	51
40 min	5732	52
60 min	6260	47

These results show that pre-incubation of the lung membranes with IgG does not increase the degree of inhibition observed in the inhibition assay. In a further experiment in which the IgG fraction was added immediately at the end of the 30 min incubation period with [^{125}I]-CYP, just before the reaction is stopped and the reaction mixture filtered, it was found that there was no inhibition, confirming that the inhibition is not simply occurring due to the presence of IgG on the Whatman G/FC filters during the filtration stage.

3.4 Immunoprecipitation of beta adrenergic receptors with sera from asthmatic subjects.

In order to show that IgG was in fact associated with the beta receptor I attempted to immunoprecipitate the beta receptor using sera from the severe asthmatic patients whose sera inhibited ligand binding.

3.4.1 Immunoprecipitation of solubilised beta adrenergic receptors.

Briefly, beta receptors from lung membranes (100μg protein) were specifically labelled with 50pM [^{125}I]-CYP, membrane receptors were solubilised with 0.5% Triton X-100 for 15 minutes at 30°C and then centrifuged at 48 000g in thick walled polypropylene centrifuge tubes for 30 min. Labelling with 50pM [^{125}I]-CYP was performed in the presence or absence of 10^{-5}M L(-) propranalol. After centrifugation the supernatant contained the solubilised receptors. The supernatant was then treated with Biobeads (0.1g/0.5ml) for 30 min at 4°C. 100μl aliquots of the solubilised receptor in the supernatant were incubated with 100μl patient serum (at a range of dilutions from 1:25 to 1:5000) for 18 hours at 4°C. Immunoprecipitation of [^{125}I]-CYP-receptor complexes was accomplished by the addition of antiserum to human IgG, which had I raised in rabbits (see Appendix C), at a 1/12 final dilution for 4 hours at 4°C and then centrifuging at 12 000g for 5 min, collecting the pellet and counting the radioactivity in a gamma counter. The concentration of solubilised beta receptors precipitated was calculated by subtracting the counts obtained in the presence of

L(-) propranalol from the counts obtained in the absence of L(-) propranalol.

Incubation of [^{125}I]-CYP with serum alone did not result in immunoprecipitation of [^{125}I]-CYP upon subsequent addition of rabbit anti-human IgG.

Applying a 1/12 dilution of rabbit anti human serum, and a range of dilutions of patient serum from 1:25 to 1:5000 (in control serum) immunoprecipitation of beta adrenergic receptors was demonstrated with only one patient's serum (VDM). Attempts to immunoprecipitate beta receptors with 10 further ligand inhibitory sera were unsuccessful (Fig. 22).

Since beta receptors with bound [^{125}I]-CYP were precipitated, these experiments suggested the human immunoglobulins were not, in fact, binding to the ligand binding domain, but to another domain of the receptor. This also suggested that by its very nature, a ligand inhibitory assay may not be the best assay for the detection of anti-beta receptor antibodies.

3.4.2 Size of solubilised labelled protein.

In order to check the size of the solubilised [^{125}I]-CYP labelled protein in 3.4.1, 140μg of guinea pig membrane was incubated with 100pM [^{125}I]-CYP in the presence or absence of L(-) propranalol for 30 minutes followed by solubilization in 0.5% Triton X-100 for 15 min and

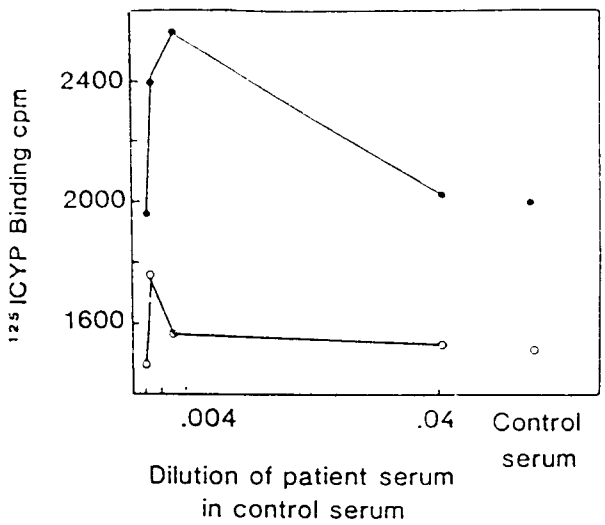


Figure 22. Immunoprecipitation of increased propranalol displacable [^{125}I]-CYP binding to solublised guinea pig beta-2 adrenergic receptors using patient serum (VDM) and a 1:12 dilution of rabbit anti human serum (see 3.4.1).

○ Binding in the presence of (L) propranalol.

● Binding in the absence of (L) propranalol.

centrifugation at 48 000g for 30 minutes. The supernatant was treated with Biobeads (0.1g/0.5ml) for 30 min at 4°C and 100µl loaded on to 1 ml spin columns using the method of Penefsky (1977) employing Sephadex G75 (retains MW 1500 to 70 000) and Sephadex G50 (retains MW 1500 to 30 000). Counts were obtained as follows (Mean of triplicates).

	with propranalol cpm	without propranalol cpm	Specific binding of solubilised protein
Sephadex G75	569	569	-
Sephadex G50	693	2232	1539

Thus the size of the solubilised [^{125}I] labelled protein was less than 70 kDa but greater than 30 kDa, consistent with the reported size of the guinea pig beta receptor of approximately 68 kilodaltons.

3.5 Western blotting of guinea pig lung membranes with patient IgG

Since both the [^{125}I]-Protein A assay (3.3.3) and the immunoprecipitation assay (3.4.1) have clear limitations, a more direct assay for antibody interaction with beta receptors is necessary. In order to see whether human immunoglobulin bound to any protein bands in the guinea pig lung membranes, polyacrylamide gel electrophoresis 11%

(PAGE) of 20μg GP lung membrane proteins was performed (Fig. 23) and proteins transferred electrophoretically on to nitrocellulose paper by Western blot (Towbin et al. 1979). After blocking overnight with 0.3% Tween and 1% foetal calf serum in Tris buffered saline pH 7.4, a dilution of DEAE purified IgG, from eight subjects, equivalent to their serum concentration of 1/200 (measured by Nephelometry) was incubated with the nitrocellulose paper under gentle agitation for 90 minutes and then washed three times with TBS-Tween. After further incubation of nitrocellulose paper with a 1/500 dilution of HRPO conjugated goat anti-human IgG and incubation with the HRPO substrate 4 chloronaphthol no bands were seen with IgG prepared from 4 asthmatics or with IgG prepared from 4 non-asthmatics. These patients included those patients who were initially positive in the Protein A (3.3.3) assay and the patient who was positive in the immunoprecipitation assay (3.4.1).

It was concluded that non-specific adherence by IgG to the proteins in guinea pig lung membranes was not occurring to guinea pig lung membrane proteins separated under reducing or non reducing conditions. However, since femptomolar binding of IgG would not be detected by immunoblotting (Towbin, personal communication), these experiments do not exclude the possibility of IgG interactions with the beta receptors at a very low concentration via other mechanisms but sufficient to interfere with [^{125}I]-CYP binding. In order to demonstrate IgG binding to receptors on Western blots, a much higher concentration of beta-receptors per lane is mandatory (picamolar to nanomolar). However, even if it is possible to obtain nanomolar concentrations of beta-2 receptors on Western blots, absence of binding of immunoglobulin fractions to

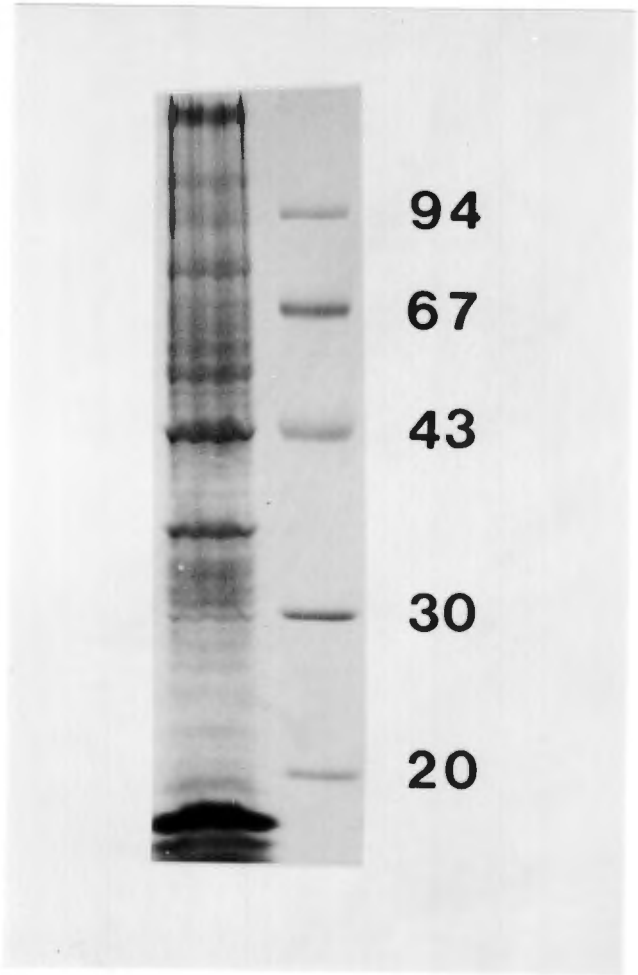


Figure 23. 11% PAGE of 20μg guinea pig lung membranes. Molecular weight markers are shown on the right.

adequate numbers of receptors may be as a result of alteration of important conformational epitopes during electrophoresis of the receptors. These problems may be eliminated if it is possible to demonstrate immunoglobulin binding to intact beta receptors on fixed or permeabilised cells. This may be possible by light microscopy if a line of cells could be obtained with expresses human beta-2 adrenergic receptors at high levels, or alternatively could be studied using a fluorescent activated cell sorter on established human cell lines.

3.6 Immunogenicity of lung membrane preparation in the New Zealand White Rabbit

Anti-beta-2 receptor autoantibodies may theoretically be induced by exposure of the immune system to intact a partially degraded beta-2 receptors either from altered self, by exposure to beta receptor ligands and the development of anti-idiotypic antibodies (Strosberg et al. 1985), or by anti-idiotypic antibodies to viruses (e.g. reovirus which bind to the beta receptor) or possibly by exposure to epitopes of intact or partially degraded beta receptors from another species in which the receptor displays homology but is not identical in its amino acid sequence (e.g. avian receptors or calf receptors in diet).

I have investigated whether direct exposure of New Zealand white rabbits to membranes prepared from calf lungs and human lungs would induce "ligand inhibiting" antibodies to beta receptors, which could be measured in a [^{125}I]-CYP binding assay. Before immunization I confirmed that the membranes prepared from calf and human lungs had

beta-receptors by [^{125}I]-CYP binding and functional cAMP assays. The immunization protocol, the course of the rabbits, the antibody responses and post-mortem findings are described in detail in Appendix D. In short, both animals developed a curious, severe wasting disease and developed antibodies to proteins in human and calf lung membrane which also cross-reacted with the rabbit lung membranes, as shown by Western blotting. There was, however, no evidence of the development of anti-beta receptor antibodies in the rabbits, as measured by [^{125}I]-CYP inhibition assay of 1:50 dilution of rabbit serum and there was no evidence of asthma in the rabbits although the lungs of both rabbits were congested and haemorrhagic at post-mortem.

While these experiments do not disprove that anti-beta receptor antibodies can be induced by exposure of an animal to foreign cell membranes containing foreign beta receptors (since a number of different immunization protocols may need to be tried to raise the desired antibodies), they do suggest that the induction of autoantibodies to the ligand binding site of beta receptor in this way is not likely to be easy. Clearly, the immunogenicity of the beta receptors in a given animal will depend partially on the amino acid sequence differences of the receptors and also on the hydrophilicity or hydrophobicity of the different domains of the receptor. Sequence differences between the human, calf and rabbit beta receptors have as yet not been studied.

3.7 Discussion.

The studies in this chapter have largely been conducted to confirm the phenomenon of ligand inhibition to beta receptors using a more specific ligand, [^{125}I]-CYP, than previously reported and a different lung membrane preparation.

I have confirmed that both serum and purified IgG fractions from asthmatic and non-asthmatic subjects influence the *in vitro* binding of the ligand [^{125}I]-iodocyanopindolol to guinea lung membrane beta receptors. Sera from 178 individuals have been studied.

In both the preliminary study of 77 subjects (54 asthmatic and 23 controls) at 1:100 dilution and in the prospective study of 101 subjects (76 asthmatics and 25 controls) at 1:50 dilution, greater than 50% inhibition of [^{125}I]-CYP binding only occurred in the asthmatic patients. In the prospective study, 88% of the asthmatic patient's sera displayed inhibitory activity which was within the range obtained with non-asthmatic sera. Significant differences between the asthmatics and non-asthmatics were not found ($p = 0.287$, Kruskal Wallis Test) at 1:50 or 1:250 dilutions of sera. (In previous analysis of this data using the Mann Whitney test and Student T tests, I did, in fact, obtain a significant difference ($p = <0.05$) between groups A and C but this was not borne out by the Kruskal Wallis test which is more suitable for this data). A marked inhibition (92%) was also obtained with a fresh serum sample from a non-asthmatic control subject, in the

experiments with cloned human beta-2 receptors in Chapter 6, again deonstrating that significant ligand inhibitory activity is not exclusive to asthmatic sera. Comparison of the patients whose sera inhibited ligand binding more than 50% also revealed no obvious clinical differences with respect to clinical course or treatment requirements when compared with the asthmatic patients whose sera were less inhibitory (detailed lung function tests were not performed).

These studies therefore do not suggest that "autoantibodies" to the beta receptor as defined by the original assays reported by Venter et al. (1980) and Fraser et al. (1981) are clinically important in asthmatic children. The laboratory observations suggest that these "autoantibodies" play a very small role, if any, in the pathogenesis of the disease. Furthermore, it is doubtful whether these immunoglobulin fractions should be called "autoantibodies" at all.

In the first instance before one can assign significance to "autoantibodies", the assays for detecting autoantibodies must be specific and reproducible. The [^{125}I]-HYP ligand binding assays used by Venter et al. (1980) and Blecker et al. (1984) while lacking a degree of specificity for the beta adrenergic receptor (Pocret, 1978). Although this was implied there was no firm evidence that the "autoantibodies" previously described, in fact, interact directly with the ligand binding domain. Conceptually, at the time of these studies, it was believed that the ligand binding domain resided in an extra-membranous location (Venter, 1983). However, since the cloning of the beta-2 receptor, molecular modelling based on the gene sequence Dixon et

al. (1987) has suggested that the ligand binding domain is not extramembranous but involved its rhodopsin-like core. This concept has been supported by my own studies on mutant human beta-2 receptors (4.3.1-4.3.6) and by those of others (Kobilka et al. 1988, O'Dowd et al. 1988, Strader et al. 1989, Chung et al. 1988). It is difficult to imagine with more recent concepts of the topography of the beta-2 receptor and its ligand binding domain, how antibodies could inhibit the binding of a small ligand such as [^{125}I]-CYP or [^{125}I]-HYP sterically, without the immunoglobulin somehow gaining access to the ligand binding pocket. I have extended this discussion in more detail in Chapter 5, in which I have addressed the theoretical possibility of an antibody binding to a ligand binding domain, by producing a monoclonal antibody to a peptide derived from the proposed ligand binding domain.

I have invested considerable experimental effort in evaluating the [^{125}I]-CYP ligand binding assay itself, in order to obtain binding data which was reproducible (Chapter 2). I have also critically re-examined the [^{125}I]-CYP inhibition assay and have found that a dose responsive inhibitory effect does not require a long (30 min) pre-incubation period, is not removed by dialysis of the serum against 20mM sodium phosphate buffer, is not destroyed by heating the serum to 56°C. The inhibitory activity is present in a DEAE purified IgG fraction and serum and is also reproducible.

While the [^{125}I]-CYP inhibition assay was reproducible, it is clear that it may not be the best assay to detect auto-antibodies to the beta

receptor since it will, by definition, exclude sera with antibodies which interact with other non-ligand binding domains. The [^{125}I] Protein A assay is non-specific and the immunoprecipitation assay, which depends on intact ligand binding for identification of precipitated receptors, will detect sera which have antibodies to non-ligand binding domains. One would, therefore, not expect a good correlation or even concordance between the results of the [^{125}I]-CYP inhibition assay and the immunoprecipitation assay.

A clear demonstration of direct interaction between human antibodies and the human beta receptor has never been reported using Western blotting or direct immunofluorescence, mainly because of the difficulties in obtaining a sufficient amount of human beta-2 receptors in the right conformation for such studies. Mammalian beta-2 receptors are difficult to purify and tend to be unstable with respect to their ligand binding characteristics, when they are outside of their normal intramembranous milieu (Cuberop and Malbon, 1984). In the past, amphibian beta receptors were preferred for molecular studies of purified beta receptors (Shorr et al. 1981). The indirect methods of demonstrating interactions between immunoglobulins and receptors were employed. The assay which seemed more promising was the immunoprecipitation assay, but since antibodies detected by this assay do not inhibit ligand binding their possible pathological significance is less clear, although antibodies to non-ligand binding domains are known to be important in the pathogenesis of myasthenia gravis.

In considering the possibility that auto-antibodies may play a role in asthma, it is critical to have some information on the sensitivity and specificity of the auto-antibody testing (Galen and Gambino 1975). Sensitivity may be defined as "positivity in disease", or the probability of a positive test given the presence of disease. Specificity on the other hand is often referred to as "negativity in health" or the probability of a negative test, in the absence of the disease in question. However, to apply these criteria one must have a reliable cut-off for a positive result for the assay being evaluated.

In evaluating autoantibody testing it may be assumed (Dawkins 1985) that:

1. A given autoantibody can be treated as a continuous variable and that it will be present in any given serum to a greater or lesser degree.
2 The test used to measure this antibody is capable of distinguishing between at least several amounts of the antibody.
3. Health is associated with less antibody than disease.
4. There will be some overlap between health and disease since the amount of autoantibody will not be the sole determinant of whether or not disease is present at that particular time.

An important consideration in applying formulae to any disease is to take into consideration the prevalence of the disease in the population as a whole. If the assumptions of Dawkins, above, are to be applied theoretically to the results of 3.2.2 using a 1:50 dilution of serum

with a cut-off of 50% inhibition as being a positive result the data may be displayed as follows:

	Positive	Negative	Total
Disease Group (Asthma)	9	67	76
Healthy Group (Control)	0	25	25
Total	9	92	101

Thus for the diagnosis of asthma, the sensitivity of the [^{125}I]-CYP inhibition assay was thus 11.8%, the specificity is 100%, the predictive value of a positive test was 100% but the predictive value of a negative test was 27%. However, this relatively small data set in 3.3.2 poses a problem in defining a positive result. Since the prevalence of asthma in Cape Town children is 7%, it is necessary to give information on approximately 1064 normal individuals if 81 patients with asthma are compared, if one is going to make reliable calculations of sensitivity and specificity.

Milgrow and Witebsky (1962) applied five criteria for judging that an autoimmune disorder is the cause of a disease.

1. A circulating antibody or a cellular immune reaction is documented in the patient with the disease.
2. A specific antigen is involved.
3. It is possible to produce an antibody in an experimental animal by immunizing with the antigen.
4. The disease should develop in the immunized animal.

5. Passive transfer of the disease is possible with serum or immunocytes.

It is immediately apparent that it would be difficult to apply the above criteria to an evaluation of anti-beta adrenergic receptor antibodies in human asthma until a pure source of human beta receptors was available for immunization in an experimental animal. An autoimmune pathogenesis to asthma based on autoantibodies to the beta-2 receptor could still theoretically exist, but there are as yet no animal studies fulfilling Milgrow and Witebsky's criteria in 3-5 above.

I have demonstrated that both crude human and foetal calf lung membranes are immunogenic in the New Zealand White Rabbit and induced a wasting disease with cross-reacting antibodies in these animals (Appendix D) but the rabbits at no stage developed any evidence of asthma. Serum from the immunized animals did not inhibit [^{125}I]-CYP binding to beta receptors in the ligand binding inhibition assay above the degree of inhibition obtained with control rabbit serum (10-35%) suggesting that the ligand domains of human and calf beta receptors were not highly immunogenic in the rabbit.

The immunogenicity of cross-species beta-receptors may better be theoretically investigated if an immunogenic quantity (micrograms) of purified receptors could be obtained, however, such a model would not represent a physiological state, since under normal conditions individuals are not likely to be exposed to such high concentrations of purified receptors out of their normal intramembranous context.

Furthermore, with recent cloning of the beta-2 receptor gene, it is clear that mammalian beta receptors are highly conserved in their amino acid sequences, particularly in the hydrophobic ligand binding domains. It is thus most unlikely that if antibodies were induced to other domains by cross species exposure, these antibodies would influence ligand binding at all, unless they induced further conformational changes in the molecule.

Sterin-Borda et al. (1984, 1988) have repeatedly confirmed that IgG in the sera of patients with Chagas disease non-competitively inhibits antagonist ligand (^{3}H-DNA) binding to rat heart B1 receptors and that Chagasic IgG induced a significant increase in cAMP levels in cardiac homogenates and spleen homogenates which was not seen with IgG from normal individuals. These findings are somewhat paradoxical in that an immunoglobulin which inhibits antagonist ligand binding, also stimulates cAMP levels suggesting that the sites of action for the two effects are likely to be different.

Reports by Limas (1989a) on the presence of autoantibodies in patients with idiopathic cardiomyopathy are remarkably similar to our own data, with respect to the degree of inhibition of ligand binding, the overlap with normal individuals and non competitive inhibitory effects of purified IgG fractions in ligand inhibition.

Recently increased and aberrant HLA DR II expression has been demonstrated in the airways of asthmatic subjects (Holgate 1990, personal communication). Analogous to Graves disease (Botazzo et al 1983)

this provides an attractive theoretical mechanism for the amplification of the production of autoantibodies to membrane proteins and particularly beta-2 receptors in the airways of allergic subjects. However, this mechanism has not yet been tested and remains speculative.

More definitive information about the mechanism of [^{125}I]-CYP inhibition by serum/IgG was obtained, in 6.6.2 using membranes containing cloned human beta-2 receptors in which I demonstrated that the [^{125}I]-CYP inhibition was non-competitive. This data confirmed that the serum inhibitory factors are not in fact competitively binding to the [^{125}I]-CYP binding site in the proposed hydrophilic pocket of the ligand binding site.

However to explore a theoretical possibility of an immunoglobulin interacting with the ligand domain, I have constructed a monoclonal antibody to a synthetic peptide derived from a proposed ligand binding domain and these experiments are described in Chapter 5.

It is relevant at this juncture, to consider and debate the possible significance of ligand inhibitory human serum. The demonstration of immunoglobulin fractions from several human subjects inhibiting [^{125}I]-CYP binding to beta adrenergic receptors in guinea pig lung membranes, may simply be an *in vitro* observation. One may postulate that immunoglobulins or other serum fractions exert some as yet unidentified and undefined role in modulating catecholamine binding to beta adrenergic receptors *in vivo*, or may modify normal regulation of

the beta adrenergic receptors in a physiological sense. Immunoglobulins may "block" or attenuate beta receptors which are normally bathed in plasma (e.g. on lymphocytes, endothelial cells, or platelets) and are continually exposed to catecholamine bursts. However, any modulatory role of such immunoglobulins on beta receptors on cells which are not normally directly in contact with plasma, e.g. brain or muscle cells, is likely to be negligible. One could postulate that this could result in a differential sensitivity of different cells (e.g. smooth muscle versus lymphocytes) to small changes in catecholamine levels, independent of the number of beta receptors expressed on each cell, since the levels of catecholamines surrounding cells which are not in the intravascular compartments, (e.g. neuronal), i.e. by simple diffusion, are likely to be much lower than the serum catecholamine levels. Receptors in direct contact with plasma are thus more likely to be in a state of down regulation than beta receptors not normally exposed to plasma, unless they are protected by other (serum) factors.

While this postulate makes physiological sense, it needs to be tested experimentally before it can be accepted, and in particular the following questions must be addressed:

1. Are the results with guinea pig membrane preparations purely cross species *in vitro* phenomena, or are they applicable to membranes from cells expressing human beta receptors?
2. Do serum or IgG fractions of sera also block beta receptor agonist activity (e.g. isoproterenol) *in vitro*?

3. What is the relationship between agonist and antagonist inhibition?
4. How are the serum blocking factors for [^{125}I]-CYP binding induced or regulated?

To answer these questions adequately the following experimental reagents are necessary:

1. A reliable source of human beta-2 adrenergic receptors.
2. A cell system expressing high levels of human beta-2 receptors in which functional activity can be measured.
3. A "control" monoclonal or polyclonal antibody specifically directed to the ligand domain of the beta-2 receptor.
4. Further purification and identification of the factors in the serum responsible for the inhibitory effects.

I have systematically attempted to address these four questions in this thesis. Chapter 4 describes the molecular biological approaches I have utilised to obtain a cell system expressing human beta receptors for further studies of the effects of serum on agonist (functional) and antagonist ligand binding which I have described in Chapter 6.

CHAPTER 4

IDENTIFICATION OF THE BETA-2 ADRENERGIC RECEPTOR MESSENGER RNA SPECIES AND STUDIES OF THE LIGAND BINDING DOMAIN USING MUTANT BETA-2 ADRENERGIC RECEPTORS.

4.1 Introduction

Extremely low levels of the beta-2 receptors normally expressed on cell membranes, i.e. femtomoles/mg protein, make direct studies of antibody interactions with the ligand domain of the receptor protein, using conventional immunological methodology (e.g. immunofluorescence, Western blotting) difficult. In the past studies demonstrating immunoglobulin binding to beta-2 receptors have used receptors, which have been affinity purified and concentrated up to 1500 fold (Caron et al. 1979, Wang et al. 1989). Affinity purification of beta-2 receptors is difficult and had only been perfected in a few major centres. One disadvantage of affinity purified beta-2 adrenergic receptors is that they are not in a functional state and the effects of serum, or other factors, on agonist stimulated receptor function cannot be studied.

With the advent of recombinant DNA technology, the prospect of obtaining genetically engineered human beta-2 receptors expressed in a mammalian cell at high levels provided an alternative approach to obtain a ready source of human beta-2 receptors for further studies necessary to answer some of the questions I raised in Chapter 3.

It was fortuitous that at the end of 1986, I was invited to join, as a Visiting Fellow, the research programme directed by Dr. Fuzon Chung, in Dr. Craig Venter's unit in the laboratory of Receptor Biochemistry and Molecular Biology at the N.I.H., Bethesda, Maryland. Dr. Chung was attempting to clone and sequence the human beta-2 receptor gene from a human neonatal brainstem cDNA library (constructed by Dr. R.A. Lazzarini, N.I.H.) in λ-gtll, using an oligonucleotide probe (51-mer) prepared from the C terminus (base pair 1384-1435) of the hamster lung beta adrenergic receptor cDNA sequence (Dixon et al. 1986). At this time the human beta-2 receptor gene had not been sequenced and the size and distribution of the mRNA species for the human gene was totally unknown. I thus decided to temporarily stop my biochemical studies with the asthmatic sera and devote some time to learning the techniques of molecular biology with the view to obtaining ultimately a genetically cloned source of human beta-2 receptors for further studies.

My initial project was to extract and identify the size of the human beta-2 receptor mRNA transcript and to study its regulation by steroids in the W1-38 human fibroblast cell line. These studies were initially difficult and tedious since RNA work had not previously been performed in Venter's Unit and I had to set up and select and evaluate RNA preparative methods on my own, before I could commence any definitive studies. These studies enabled me to construct sense and anti-sense riboprobes of the beta receptor. Once the human beta-receptor gene was cloned, sequenced and had been expressed in B82 mouse fibroblasts, I attempted to purify human beta-2 receptors, for

further antibody studies, from the fibroblasts, using alprenalol affinity chromatography, but the low yields of receptor I obtained were not suitable for antibody studies. Dr. Fuzon Chung in the mean time had made a full length cDNA beta-2 receptor probe designated CLVF-108.

The intriguing idea that allergic subjects may have a genetic defect in beta-2 receptor structure, makes the *in vitro* examination of theoretical genetic models of dysfunctional beta-2 receptors most interesting. The opportunity to do this using site-directed mutagenesis arose. I therefore decided to work with Chung on mutant beta-2 receptor construction and characterization, since this provided and exciting opportunity to learn more about the nature of the ligand binding site and to examine the *in vitro* behaviour of dysfuntional beta-2 receptors.

In an attempt to identify the residues important for ligand binding and to select mutants for further studies, I conducted the initial ligand binding studies on nine mutant beta-2 receptors constructed by Dr. Chung and then proceeded with more detailed characterization of the Aspartate 79 mutant (Chung et al. 1988) and the Asn 6 glycosylation mutant. I also attempted, while at N.I.H., to express the wild type beta-2 receptor in prokaryotic cells using the Pttq expression vectors developed by Stark, Leicester U.K. (1987) but did not at any stage identify the expected fusion product on polyacrylamide gels.

The expression vectors and probes constructed in these studies enabled me to extend my early studies with human sera (described in Chapter 3) to an evaluation of the effects of serum on the function of cloned human beta-2 receptors expressed in B82 cells (described in Chapter 6), to make and evaluate a monoclonal antibody to a peptide derived from the beta-2 adrenergic receptor sequence (described in Chapter 5) and to study a polymorphism of the human beta-2 receptor in allergic subjects, (described in Chapter 7).

4.2 Identification of the mRNA transcript of the human beta-2 adrenergic receptor

4.2.1 Isolation of RNA

Total cellular RNA was isolated using a Cesium chloride gradient according to the method of Chirgwin et al. (1979).

Fresh tissues from guinea pig lung, rat epididymal fat pad (±1gm), murine B82 cells (Gilman 1973), W1-38 human lung fibroblasts and Va_4 cells (± $5x10^7$ cells) (Hayflick 1961) (see 4.2.4) were dispersed in 4 ml of 4M guanidinium isothiocyanate, 5mM sodium citrate (pH 7.0) 0.1M β-mercaptoethanol and 0.5% sarkosyl. RNA pellets were prepared on cesium chloride gradients using the method of Maniatis (1982). RNA pellets were dissolved in 10mM Tris-HCl pH 7.4, 5mm EDTA, 1% SDS, and extracted twice in 4:1 mixture of chloroform:1 butanol. The two aqueous phases were combined and the RNA recovered by centrifugation after precipitating with 0.1 volume of 3M sodium acetate (pH 5.2) and

2.2 volumes of ethanol at -20°C for 4 hrs. Pellets were dissolved in H_2O, re-precipitated with ethanol and stored at -70°C.

I prepared Poly(A)$^+$ RNA using two methods. Initially Hybond^{TM}MAP (messenger-affinity paper) Amersham (Code RPN 1511) was employed. In this method, crude RNA is spotted on to Hybond-MAP, washed twice in 0.5M NaCl (to remove free RNA), rinsed in 70% ethanol and then washed in distilled H_2O at 70°C which releases the Poly(A)$^+$ RNA into the H_2O. Although good yields of Poly A+RNA were obtained from MAP, I did not obtain good signals when slot blots or Northern blots of Poly(A)$^+$ RNA prepared by this method were probed. I therefore purified mRNA by Oligo(dT) cellulose chromatography.

Oligo(dT)-cellulose Type 3 (Collaborative Research Incorporated, Bedford, MA, Cat. 20003) was used and the columns prepared essentially according to the protocol of Davis, Dibner and Battey (1986). Strict attention was directed to maintaining an RNase-free environment at all times. Gloves were worn, all glassware was baked at 250°C, diethyl pyrocarbonate treated (DEPC)(Sigma) water was used and wherever possible, disposable plastic/polycarbonate containers were used.

4.2.2 Agarose gels and Northern blots

(i) Glyoxal gels.

Based on the methods published by Thomas (1983), 10-20μg total RNA or 2-3μg Poly(A)$^+$ RNA was heated at 50°C for 1 hour in 1M glyoxal in the presence of 50% DMSO prior to electrophoresis on a 11 x 14cm

horizontal gel in 1% agarose (molecular biology grade) in 10mM sodium phosphate buffer (pH 7.0) with xylene cyanol and bromophenol blue as dye markers. After electrophoresis at 100V for 2 hours, by which time the dye had migrated 9 cm, gels were stained in the dark for 15 mins in 5μg/ml ethidium bromide in H_2O and destained in H_2O in the dark for 1-2 hours. RNA ladder (BRL, Gaithersburg) was used as a molecular weight marker.

(ii) Formaldehyde gels.

Dry RNA samples (10-20μg total RNA or 2-3μg Poly(A)$^+$ RNA were dissolved in 2.2μl buffer A (294μl 0.5M MOPS (pH 7.0)/0.01M EDTA(pH 7.5) and 706μl H_2O) to which was added 4.8μl formaldehyde/formamide (final concentrations of 2.2M formaldehyde and 50% formamide), heated at 70°C for 10 minutes before quenching on ice. 1.5μl of gel loading buffer (xylose cyanol/bromocresol green/sucrose) was added prior to electrophoresis on an 11 x 14cm horizontal submarine 1% agarose gel (prepared by mixing 0.5g agarose, 5 ml 10xMOPS/EDTA buffer and 36 ml H_2O after which the agarose was allowed to cool to 60°C and 9 ml 37% formaldehyde (2.2M) was added before pouring the gel). After running for 2 hours at 100V, gels were stained in the dark with 5μg/ml ethidium bromide in H_2O and destained in the dark for 2 hours. The bands were visualized using a short wave U.V. transilluminator.

I found that formaldehyde gels gave much less background staining with ethidium bromide than glyoxal gels and thus selected formaldehyde gels for further studies. The need for prolonged staining and destaining

was also circumvented by the addition of ethidium bromide directly to the sample buffer just before RNA was loaded on to the gel.

For hybridization of RNA species with beta-2 receptor cDNA or riboprobes, RNA was either applied directly onto nitrocellulose paper using slot blots (BRL Hybrislot, Gaithersburg) and dried at 80°C in a vacuum oven, or Northern blotted (Davis, 1986; Thomas 1980) on to Hybond N (Amersham) followed by U.V. irradiation. After prehybridization for 1-4 hours in 50% formamide 50 mM $NaPO_4$ pH 6.5, 5 x SSC, 0.1% SDS, 1mM EDTA, 0.05% Ficoll, 0.05% Polyvinyl pyrrolidone (PVP), 200μg/ml salmon sperm DNA and 5 mg/ml Yeast tRNA (Melton et al. 1984) slot blots or Northern blots were probed with nick translated cDNA probe CLFV 108 (bp 170 of the 5' coding region to 30 bp of the 3' untranslated region of the human beta-2 receptor gene)(Chung et al. 1987). Nick translations were performed using the Amersham Nick translation kit [^{32}P] specific incorporation: ~ 10^{-8}cpm/μg DNA.)

Filters were hybridised overnight (16 hours) at 45°C and then washed 3-5 times (20 minutes each) at 65°C in 0.1 x SSC, 0.1% SDS (pre-heated to 65°C. For some blots higher temperatures (up to 80°C) were required to remove background binding during the washing stage and with riboprobes, blots were hybridised at 65°C.

4.2.3 Identification of the human beta-2 adrenergic receptor mRNA species.

When I commenced these studies the size and distribution of the human beta-2 receptor mRNA species was unknown. My initial goal was to identify the human beta-2 receptor mRNA species in order to extend the developmental studies of the guinea pig foetal lung I had commenced in Chapter 2 and to investigate whether the level of induction of beta-2 receptors by steroids previously reported by Fraser et al. (1980) was at the level of mRNA transcription.

Total RNA was prepared (using the guanidinium isothiocyanate/cesium chloride method) from frontal lobe of human brain (stored at -70°C) (supplied by Dr. C. Venter, N.I.H.), fresh foetal guinea pig lung (54 day gestation) and adult lung tissues, and mRNA was purified by oligo(dT) chromatography. Several agarose gels separating 20μg foetal and 20μg adult guinea pig lung total RNA were Northern blotted and probed with CLFV-108 nick translated beta-2 receptor gene probe. In my initial studies, weakly positive hybridisation signals were seen as diffuse bands about 2.0-2.6 kb on autoradiography of blots of crude RNA from both adult guinea pig and human brain and no signal was obtained with foetal guinea pig lungs (Autoradiography -70°C for 18 hrs to 30 hrs). This result was promising but difficult to reproduce.

I then embarked upon a series of experiments of a trouble shooting nature, to attempt to obtain good yields of clean RNA. RNA was

prepared using alternative protocols described by Morrison (1981) using Cesium chloride, Parker, (personal communication 1986, UCT), and the rapid methods published in Davis (1986).

With each method Poly(A)$^+$ RNA was prepared using Amersham MAP paper and oligo(dT) chromatography. Each of the above methods yielded undegraded RNA as visualised on agarose gels, but autoradiography of Northern blots of up to 50μg of crude RNA and 10μg Poly(A)$^+$ RNA from adult guinea pig lungs probed with CLFV-108 were negative. I also probed these Northern blots with a 5' end labelled 51 mer oligonucleotide (C-terminal) probe (base pair 1384-1435) derived from the hamster lung B-adrenergic cDNA sequence (which was used by Dr. Fuzon Chung to isolate the human beta-2 receptor gene), but was repeatedly unsuccessful in identifying a clear mRNA species even when hybridization times, stringency washes and autoradiographic exposures were varied and dextran sulphate was added to the hybridization mixture. I also attempted to get rid of any DNA contaminating the RNA preparations by treating with RNAse free DNAse and lithium chloride (2.5M x 4hr at 4°C) and to improve the Northern blots by omitting ethidium bromide staining of the RNA before transfer.

Failure to identify the mRNA from the human brain or guinea pig beta-2 receptor gene using the above methods suggested that the beta-2 receptor mRNA was a rare species, or that more than 10μg Poly(A)$^+$ RNA was necessary, or that it was unstable and had a short half life in the organs studied. I therefore decided to study the human beta-2 receptor mRNA in a more controlled *in vitro* system using Va4 and

Wl-38 cells, since beta-2 receptors were previously found to be steroid inducible in these cells by Fraser and Venter (1980b), but the level of induction was not known.

As an alternative to Northern blots, I attempted to identify the beta-2 receptor mRNA using the BRL Hybrislot apparatus. In these experiments, 1-16μg of Poly(A)$^+$ RNA (which I had prepared from Wl-38 cells, Va-4 cells, guinea pig lung, rat heart rat adipose tissue, and human cerebellum was applied on to wetted nitrocellulose (Schleicher and Schuell), dried in a vacuum oven at 80°C, denatured in formaldehyde, prehybridised for 4 hours and then hybridised at 45°C for 10 hours with nick translated CLFV 108 probe, washed with 1 x SSC,0.1% SDS at 60°C for 1 hour and then autoradiographed for 4 hours.

Good sharp hybridization signals were obtained on the autoradiographs of the slot blot with mRNA prepared from Va4 cells (an SV40 transformed subline of Wl-26 cells) and human cerebellum (Fig. 24) but no signals, or very weak signals, were obtained from mRNA prepared from adult guinea pig lung, foetal guinea pig lung, rat heart and rat adipose tissues.

These results were encouraging and suggested that, in my hands, beta-2 adrenergic receptor mRNA studies were more likely to succeed using cells grown in tissue culture than with mRNA prepared from organs. Furthermore, if the beta-2 receptor mRNA was, in fact, such a rare species in organs, it was more likely to be detected using an

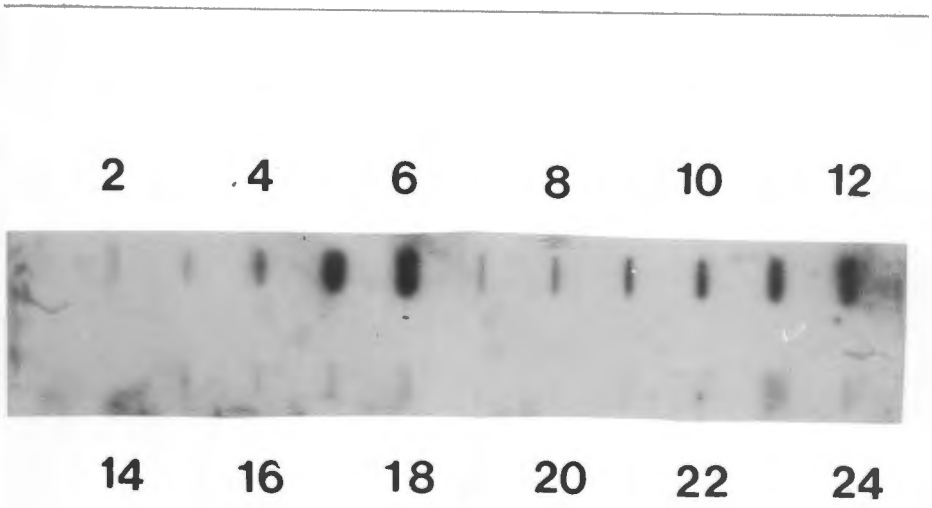


Figure 24. Autoradiograph of slot blots to which increasing amounts (1-16μg) of poly(A)$^+$ RNA from human cerebellum (1-6), Va_4 lung fibroblasts (7-12), adult guinea pig lung (13-18), and foetal guinea pig lung (19-24) was applied and probed with nick translated CLFV 108 human beta-2 receptor probe.

anti-sense riboprobe of the beta-2 receptor gene. I thus proceeded to develop and study riboprobes of the beta-2 receptor gene.
I reasoned that construction of a sense beta-2 receptor RNA strand could serve as a quantitative and qualitative control (or reference) for the natural beta-2 receptor mRNA in both the slot blots or Northern blots.

Initial studies with riboprobes were conducted with a 2.6kb fragment of the beta-2 receptor gene sequence. The 2.6kb riboprobe was derived from a Pvu-II fragment of a genomic clone LCV 517 (Chung et al. 1987) which had been cloned into pGEM-3Z with the assistance of Dr. Ulrich Lentes.

Using a protocol adapted from the Promega Biotec catalogue (1985/1986, DB4-5) I synthesised cold sense and anti-sense RNA transcripts of the human beta-2 receptor gene, and confirmed on agarose gels that the molecular size of the transcripts were 2.6kb (Fig. 25). Limits of detection of a single RNA transcript by Northern blots were then investigated. Sense and anti-sense RNA transcripts were denatured (according to 4.2.2(ii)) run on agarose gels and Northern blotted on to Hybond N. Autoradiographs of sense and anti-sense 2.6kb RNA transcripts on Northern blots probed with cDNA CLFV-108 probe are shown in Fig. 26. The cDNA probe detected as little as 5ng of sense RNA and 5 pg of anti-sense RNA on the Northern blots. Reprobing the same blots after stripping the CLVF-108 probe, with a shorter anti-sense riboprobe (326bp) which I constructed (see section 4.2.6) showed clear hybridization to only the sense RNA (down to 500pg RNA)

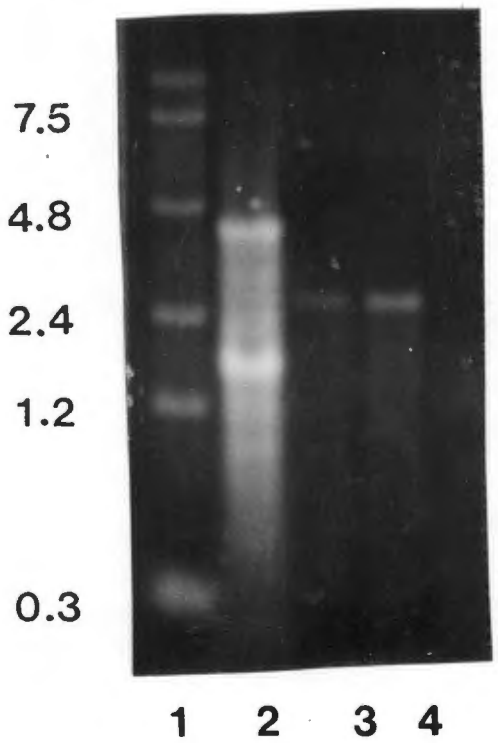


Figure 25. Agarose gels stained with ethidium bromide and photographed under U.V. light showing RNA ladder (lane 1), calf ribosomal RNA (lane 2), sense (lane 3) and anti-sense (lane 4) 2.6 kb RNA transcripts.

after 20 min at room temperature. No hybridization was detected with the anti-sense RNA on the same autoradiograph (Fig. 27) confirming the specificity of the riboprobe and a 10-fold increased sensitivity in detection of the sense RNA strand over the CLVF-108 cDNA probe. Longer periods of autoradiography (-70°C x 12 hrs) enabled detection of as little as 20 femtograms of sense and anti-sense beta-2 receptor RNA transcripts with the cDNA probe.

A source of cold sense beta-2 receptor RNA proved to be extremely useful as a control transcript for the Northern blots, which I also used as a reference from which to calculate the size of the human beta-2 receptor mRNA species in the W1-38 cells. Poly(A)$^+$ RNA was again prepared from adult guinea pig lung (40μg), foetal guinea pig lung 54 days (50μg), B82 cells (100μg), W1-38 cells (30μg) and run on an agarose gel, with calf ribosomal RNA (20μg) as a negative control and, 5μg sense and 5μg anti-sense 2.6kb beta receptor RNA as positive control transcripts. A Northern blot probed with cDNA probe, CLFV-108, overnight, washed down to a stringency of 0.1% SDS, 0.2xSSC at 45°C and autoradiographed by exposure to Kodak XAR is shown in Fig. 28.

Autoradiography for 20 min at room temperature produced sharp signals of the 2.6kb sense and anti-sense transcripts. On further autoradiography at -70°C for 4 hours a clear mRNA species 2.2-2.4 kb was identified in the lane containing 30μg Poly(A)$^+$ RNA from W1-38 cells (dexamethazone pulsed x 24 hrs) but no positive signals were obtained in any of the other lanes (other than the control sense and anti-sense RNA species) on further exposure at -70°C. Reprobing the

blots with the 2.6kb anti-sense riboprobe failed to detect any beta-2 receptor mRNA species in the guinea pig lungs but was associated with high non-specific binding of the riboprobe to the Hybond which was difficult to remove even at high stringency and at high temperatures (80-90°C) for prolonged periods (2-3 hrs). I prepared several further Northern blots with guinea pig Poly(A)$^+$ RNA which I probed with the 2.6kb riboprobe but, disappointingly, high background binding of the probe to the Hybond remained a problem.

These studies demonstrated that the size of the mRNA for the human beta-2 receptor gene was 2.2-2.4 kb. This had not been previously reported. The difficulties I experienced in detecting the beta-2 receptor mRNA species were also alluded to by Kobilka et al. (1987a) when they mapped the beta-2 adrenergic receptor to q31-q32 on Chromosome 5. These workers required as much as 90μg of Poly(A)$^+$ RNA from human placental tissue to obtain a signal on a Northern blot and commented on the rarity of the beta-2 receptor messenger RNA species.

The technical difficulties I experienced in attempting to identify and study the mRNA of the guinea pig beta-2 receptor gene using Northern blotting made the feasibility of pursuing studies of mRNA induction in the developing guinea pig lung questionable, since it was clear that large amounts of Poly(A)$^+$ RNA would be required from the foetal lungs. Since repeated attempts to clearly identify the mRNA of the beta-2 receptor in adult guinea pig lungs with the riboprobe were also negative, further studies of mRNA levels during the foetal lung ontogeny were abandoned. Clearly methods such as solution

Figure 26. Autoradiograph of a Northern blot of sense and anti-sense 2.6 RNA transcripts probed with cDNA CLFV-108 probe.
Exposure Kodak XAR film -70°C for 6 hours.
RNA standards: Lane 1 (2μg) Lane 2 (1μg) Lane 3 (0.5μg) Lane 4 (50ng) Lane 6 (500pg) Lane 7 (5pg) Lane 9 (2μg) Lane 10 (1μg) Lane 11 (0.5μg) Lane 12 (50ng) Lane 13 (5ng) Lane 14 (500pg) Lane 15 (5pg).

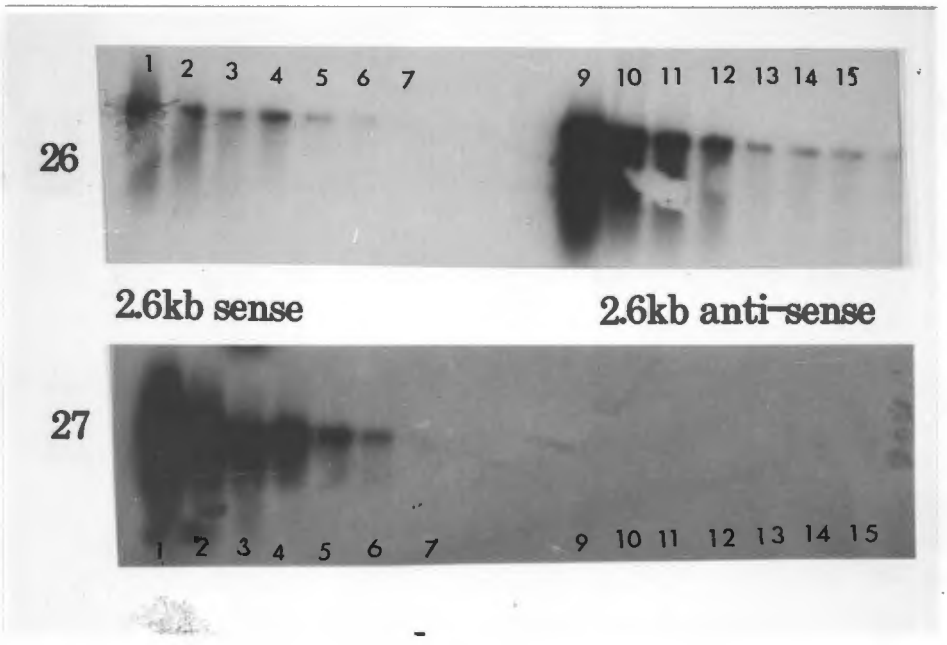


Figure 27. Autoradiograph of Northern blot (in Fig. 26) after stripping of CLFV-108 probe and reprobing with 326bp anti-sense riboprobe confirming the specificity of the anti-sense probe for the sense 2.6kb RNA transcript.

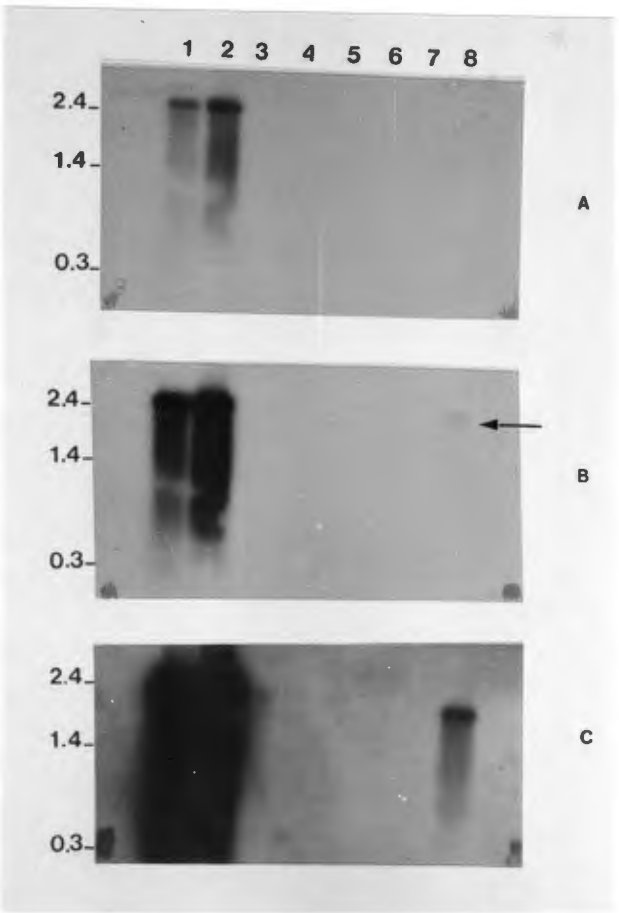


Figure 28. Autoradiograph of Northern blot of 5μg 2.6kb sense RNA (lane 1), 5μg 2.6kb anti-sense RNA (lane 2), crude RNA from adult guinea pig lung (lane 3), Poly(A)$^+$ RNA from adult guinea pig lung (40μg)(lane 4), Poly(A)$^+$ RNA from foetal (54 day) guinea pig lung (50μg)(lane 5), Poly(A)$^+$ RNA from B82 cells (100μg), crude RNA (50μg) from W1-38 cells (lane 7) and Poly(A)$^+$ RNA from W1-38 cells (30μg) probed with CLFV-108 cDNA probe at -70°C for 20 min (A)(lane 8), showing recognition of sense and anti-sense transcripts. At 4 hours (B) in addition, the 2.3 kb human beta-2 receptor mRNA transcript is just identifiable and is shown clearly by 10 hours (CA in lane 8. RNA markers (kb) using BRL RNA ladder are shown on the left.

hybridization (RNAse protection assays) would have to be explored, a shorter riboprobe (less than 500bp) would have advantages over the 2.6kb riboprobe, and *in situ* hybridization should also be considered as a technique to investigate induction of the developing guinea pig mRNA of the beta-2 adrenergic receptor. Rarity of the beta-2 receptor mRNA species has been also alluded to by Dixon et al (1986) and confirmed by Hadcock and Malbon (1988a).

Since I was able to detect the human beta-2 receptor mRNA using W1-38 cells grown in culture, (in the presence of dexamethazone), I embarked on a series of experiments to study the timing and intensity of the induction of human beta-2 receptor mRNA following pulsing with dexamethazone.

4.2.4 Induction of beta-2 receptor mRNA by dexamethazone

The ability of dexamethazone to induce beta-2 receptor mRNA in cultured cells was studied using 2 cell lines: the W1-26 Va4 human lung SV40 transformed line (ATCC CCL 95.1) and the W1-38 cell line (ATCC CCL 75).

The Va4 cells are an SV40 virus transformed derivative of W1-26 cells, a human diploid cell line established from the embryonic lung tissue of a male Caucasian.

The W1-38 cells are a human diploid cell line derived from normal embryonic (3 month gestation) lung tissue of a Caucasian female. W1-38 cells have a doubling time of 24 hours (Exp. Cell. Res. 37:614, 1965).

Cells were grown in Ham's F12 media (Gibco) supplemented with 10% fetal calf serum in an atmosphere of 95% air, 5% carbon dioxide and studies were conducted with W1-38 cells which had less than 40 population doublings. It has been previously shown that incubation of Va4 or W1-38 cells with hydrocortisone results in a 100% increase in [^{125}I]-HYP specific binding (Fraser 1980) and that this increase is dependent upon new protein synthesis. In those studies W1-38 cells showed an approximate doubling time of [^{125}I]-HYP specific binding within 48 hours with an ED_{50} for hydrocortizone of 3×10^{-7}M and a maximum response evident within 24 hours.

For the RNA studies, cells were grown to semi-confluence, fresh medium was added and cells stimulated with 10^{-6}M dexamethazone were compared with control cells. [^{125}I]-CYP ligand binding assays conducted on the cells at time intervals between 0 and 48 hrs confirmed the induction of beta adrenergic receptors by steroids previously published by Fraser (1980).

Cell membranes were prepared by homogenization of hypotonically lysed cells, using a dounce glass homogeniser (Wheaton) (20 strokes) in 5mM $NaPO_4$ pH 7.4 1 mM $MgSO_4$ (at 4°C) followed by centrifugation at 1000g x 10min to remove intact cells and nuclear debris. The supernatant was centrifuged at 40 000g x 30min (using a Beckman 70 Ti rotor) and the membrane fraction in the pellet resuspended in 20mM $NaPO_4$ 2mM $MgSO_4$ pH 7.4 buffer at a protein concentration of 3-4mg/ml and stored at -70°C.

RNA was prepared by the guanidinium isothiocyanate method (section 4.2.1). At each time point cells were scraped off the dish using a 'rubber policeman', into guanidinium isothiocyanate and RNA isolated using the cesium chloride gradient method of Chirgwin et al. (1979). Poly(A)$^+$ RNA was prepared by oligo(dT) chromatography of the crude RNA and RNA concentrations calculated by measuring the optical density by U.V. absorption spectrophotometry at 260nm. Purity of the RNA was assessed by determining the ratio of O.D. at 260nm to O.D. at 280nm.

To investigate optimal concentrations of RNA for slot blotting, crude RNA (0.06μg to 2μg) was blotted in 50μl on to Hybrislot apparatus (BRL, Gaithersberg) and probed with nick translated cDNA probe CLFV 108. Good positive hybridization signals were obtained with 1μg mRNA from W1-38 cells and the dose responsive effect of dexamethazone 10^{-9}M, 10^{-8}M and 10^{-6}M on the intensity of the hybridization signal was measured using densitometry. (Fig 29).

In the definitive experiments both Va4 and W1-38 cells were studied and steroid induction of mRNA was studied at a concentration of 10^{-6}M dexamethazone.

Va4 cells were pulsed with 10^{-6}M dexamethazone at 0 hours and mRNA prepared from pulsed and control cells at 2.5 hrs, 4 hrs, 6 hrs, 8 hrs, 12 hrs and 24 hrs. 1μg mRNA (denatured in formaldehyde) was slot blotted onto nitrocellulose, vacuum dried (80°C) for 2 hours and probed with nick translated [α-^{32}P]dCTP labelled cDNA probe CLFV 108 (specific activity 10^{-8}cpm/μg) for 8 hrs, successively washed with 2 x SSC, 0.1% SDS at room temperature and 0.2 x SSC 0.1% SDS at 45°C for 2 hours and exposed to Kodak XAR film for 16 hours at -70°C.

Autoradiographs (Fig. 30) of the Va4 cells showed an increase in mRNA signal at 4 hrs which was sustained through 24 hrs in the dexamethazone stimulated cells but not in the control cells. Similarly, autoradiography of the Wl-38 cell RNA slot blots studied at 4 hrs, 8 hrs and 24 hrs showed a slight increase of mRNA at 4 hrs, but a marked increase was noted at 8 hrs which was sustained at 24 hrs in the dexamethazone pulsed cells but was not seen in control cells (Fig. 31). No positive signals were obtained with Poly(A)$^+$ RNA from guinea pig lungs on the slot blots.

Several experiments were conducted to demonstrate a dexamethazone induced increase in the 2.2-2.4 kb mRNA species on Northern blots. Although both the 2.6kb riboprobes and cDNA probes of the beta-2 receptor gene were employed and as much as 50μg Poly(A)$^+$ RNA transferred on to the Northern blots, at the time points studied, an increase in mRNA was not demonstrated on the Northern blots (4 hrs, 6 hrs, 18 hrs, 24 hrs). High background binding occurred with the 2.6kb riboprobe. I decided to construct a shorter riboprobe to reduce background. It also seemed that solution hybridization with RNAse protection may be a better alternative approach to measuring the dexamethazone induced RNA of the beta-2 receptor gene. This has subsequently been successfully achieved by Hadcock and Malbon (1988).

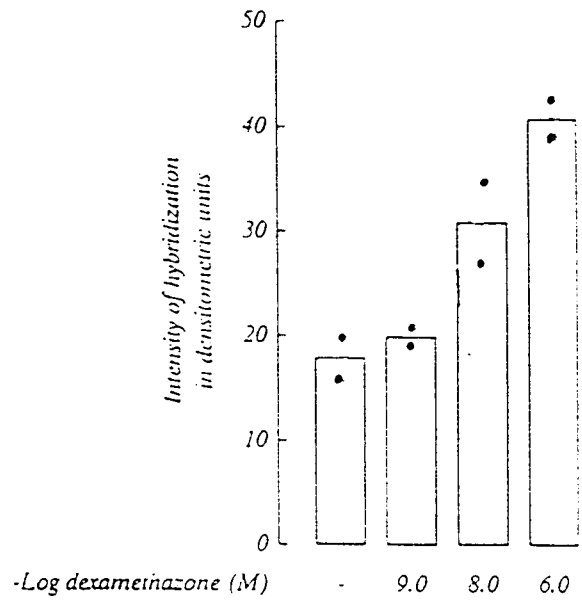


Figure 29. Intensity of hybridization of CLFV Nick translated probe to 1μg poly(A+)RNA from Wl-38 cells pulsed overnight with 10^{-9}M, 10^{-8}M and 10^{-6}M dexamethazone. Autoradiographic signals were measured by densitometry and are expressed in densitometric units. Bar values represent the mean of duplicate assays (•).

The slot blots suggested, for the first time, that the beta-2 receptor expression could be transcriptionally regulated by steroids and I reported this at the 1987 FASEB congress (Federation Proceedings Vol. 46:4, 1463 April 1987, Washington). However, subsequent attempts to demonstrate an increase in mRNA transcription on the Northern blots were unsatisfactory and clearly further studies which included negative internal RNA reference/standards (e.g. β actin) were indicated. I was unable to explain the relative ease with which increases in binding of the probe to mRNA from dexamethazone pulsed cells on slot blots was demonstrated at high stringency using small amounts of poly(A)$^+$ RNA, (1μg), but not readily demonstrated on Northern blots. It is possible that these hybridization signals could have been non-specific although they were highly reproducible and dose responsive.

A year later, Collins et al. (1988) successfully demonstrated steroid inducible β-2 adrenergic receptor mRNA by Northern blot hybridization using Hamster vas deferens cells (DDT_1 MF-2) and reported that a peak of β-2 adrenergic receptor mRNA was reached within 1-2 hours. These cells demonstrated a peak of beta-2 adrenergic receptor induction between 6 and 12 hours, which is more rapid than the rate of increased receptor expression reported in the Wl-38 cells.

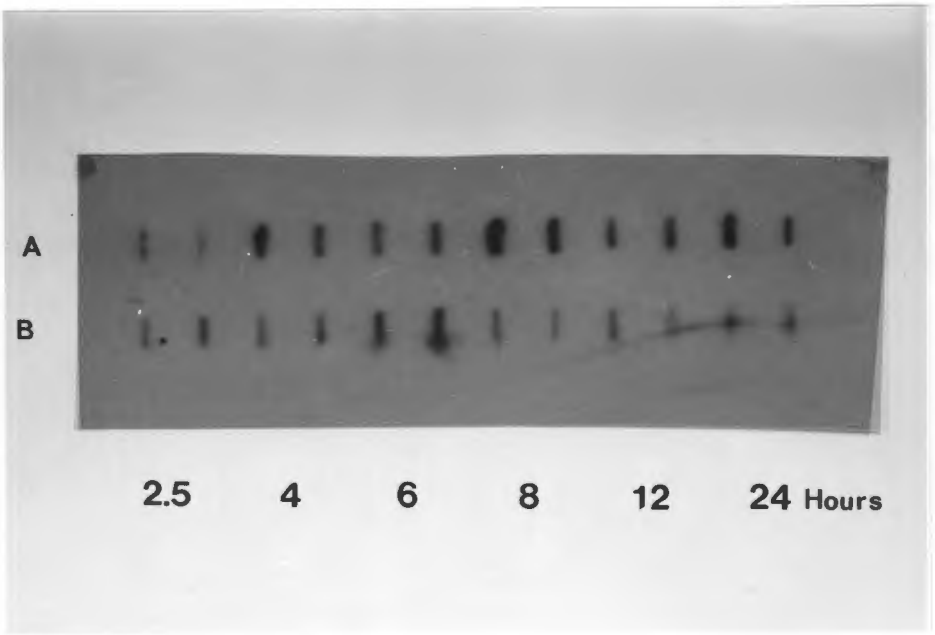


Figure 30. Time study of the induction of human beta-2 receptor mRNA in Va4 cells. 1μg poly(A)$^+$ RNA prepared from Va4 cells (in duplicate)(harvested at 2.5, 4, 6, 8, 12 and 24 hours after pulsing with 10^{-6}M dexamethazone)(A) or control cells (not pulsed with dexamethazone)(B), slot blotted on to nitrocellulose paper and probed with [α^{32}P]dCTP nick translated CLFV-108 probe. Stringency 0.2xSSC and 0.1%SDS at 45°C for 2 hours. Exposure Kodak XAR, -70°C for 16 hours. A sustained increase in uptake of the probe is seen in the dexamethazone pulsed cells. A transient increase in uptake of the probe was also observed in control cells at 6 hours.

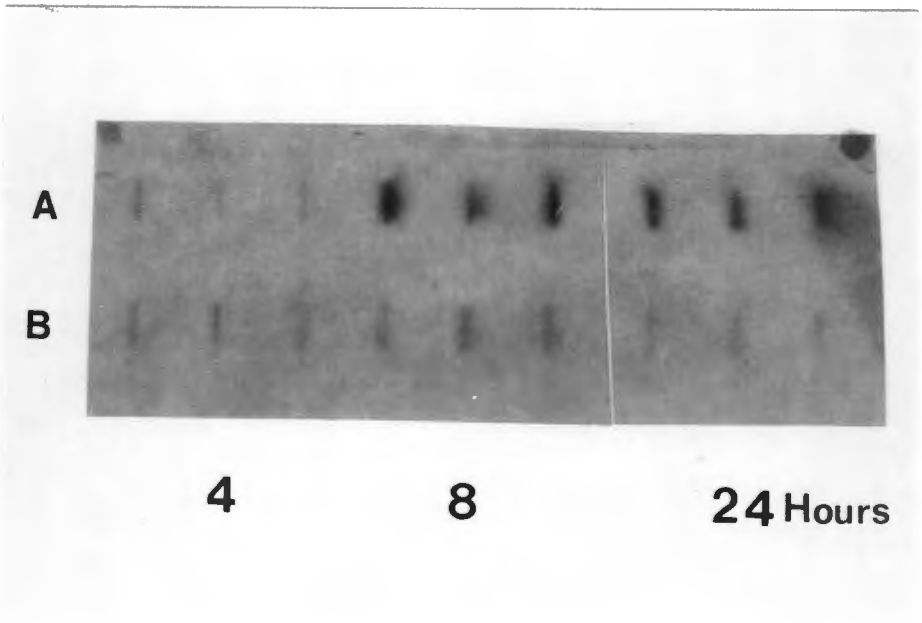


Figure 31. Time study of the induction of human beta-2 receptor mRNA in W1-38 cells. 1μg poly(A)$^+$ RNA (in triplicate) prepared from W1-38 cells harvested at 4, 8 and 24 hours after pulsing with 10^{-6}M dexamethazone (A) or controls without dexamethazone (B). Stringency 0.2xSSC and 0.1% SDS at 45°C for 2 hours. Exposure Kodak XAR -70°C for 16 hours.

Using solution hybridization Hadcock and Malbon (1988), studying the DDT_1 MF-2 hamster vas deferens cells have shown an increase of steady state beta adrenergic receptor mRNA from 0.37 pg mRNA per μg total cellular RNA in untreated cells to 1.05 pg of mRNA per μg of RNA in cells treated with dexamethazone for 2-4 hrs, followed by a sharp decline by 6 hours to a level approximately twice that of untreated cells. The half life of the receptor mRNA in the presence of actinomycin D was found to be 12 hrs in both untreated and dexamethazone-treated cells. Even at low concentrations of dexamethazone (1 nM), significant increases in mRNA were observed.

In studies of beta receptor mRNA in S49 mouse lymphoma mutants, Hadcock et al. (1989) showed that cAMP dependent protein kinase activity, and functional receptor coupling to GS was required for agonist induced down regulation of beta-2 receptor mRNA. In these studies the steady state of levels of mRNA were 1.72 $\pm$ 0.08 attomoles/μg total cellular RNA. Hadcock and Malbon (1988b) have also demonstrated that cholera toxin and forskolin treatment of DDT_1 MF-2 cells also down regulated beta receptor mRNA. More recently Bouvier et al. (1989) have demonstrated that cyclic AMP exerts a feedback control of beta-2 receptor number associated with a reduction in steady state mRNA levels.

Transient mRNA accumulation and transcriptional enhancement has been reported for other steroid regulated genes e.g. rat phosphoenolpyruvate carboxykinase, and rat tyrosine hydroxylase (Tank 1986).

The ability of the beta receptor gene to respond to glucocorticoids also has a sound structural basis. Four consensus sequences for glucocorticoid responsive elements have been identified in the cloned beta-2 adrenergic receptor gene sequence by Chung et al. (1987), Kobilka et al. (1987) and Emorine et al. (1987): two in the coding region, one in the 3' non-coding region and one in the 5' upstream region. The functional significance of each of these glucocorticoid responsive elements has not yet been elucidated. Studies by Samuelson and Davies (1984) showed that hydrocortizone could reverse prior agonist induced uncoupling of beta-adrenergic receptors in neutrophils, without an observable effect on down regulation. It is apparent that the effects of steroids on beta-2 receptor number and function may be dependent on cell type, prior agonist treatment, or the state of down regulation, and it is likely that the beneficial effects of steroids in the airways of asthmatic subjects may also be due to effects at levels other than that of gene transcription of the beta-2 receptor.

4.2.5 Construction of a short 326bp riboprobe of the human beta-2 adrenergic receptor.

A shorter riboprobe would have several potential advantages over the 2.6kb riboprobe. It would be useful in the future for *in situ* hybridization and should also give less background on the Northern blots than the 2.6kb probe. I selected a 326bp segment derived from a Kpn-1- Pst-1 digest of the coding region of the beta-2 receptor gene. A cDNA probe derived from this 326bp piece was also found to be most useful for studies I conducted to identify a probable site of the reported polymorphism of the human beta-2 receptor gene. (see Chapter 7). I also used a riboprobe of this 326bp segment to check the authenticity of the sense and anti-sense 2.6kb RNA transcripts which I constructed in 4.2.4.

I obtained the 326bp piece by digesting a PVU 11 fragment of the genomic DNA LCV 517 which had been cloned into PSMG, with Pst-1 and Kpn-1 (373 bp-699 bp). The predicted 326bp piece was identified on an agarose gel (Fig. 32), purified by electroelution from the gel and ligated into the multiple cloning site of pGEM-3Z which had been previously digested with Kpn I and Pst 1. Electroelution was performed by cutting out the 326bp band in the agarose placing it in 1xTBE in Spectropor membrane tubing and applying a voltage of 150V for 30 min. DNA moved to the positive electrode; the polarity was then reversed for 30 seconds and the DNA transferred to a fresh tube. 10μg of tRNA and an equal volume of n-butanol was added. After centrifugation for 10 min the infranatant was collected and extracted with phenol/chloroform, before precipitation in ethanol:sodium acetate. After air drying the precipitate was suspended in dd H_2O.

For the ligation reaction, 4μg of Kpn-1-Pst-1 digested DNA (concentration 1.7 μg/μl) was incubated with 5μg pGEM-3Z, 4μl (5x ligation buffer), 2μl T4 ligase and 5μl dd H_2O.

To transform the competent cells, 3μl of the ligation mix was added to 300μl of competent TG-1 E. coli cells made competent according to the method published in Davis et al. (1986) in 50mM calcium chloride after glycerol had been removed, incubated at 4°C for 30 min and then heat shocked at 42°C for 2-3 min. 0.7 ml of warm 2X TY medium was added and after 30 min incubation at 37°C plated on to L Ampicillin plates (50, 100 or 300μl in 2.5 ml soft agar (spiked with 40μl Xgal, 40μl ITPG and 25μl (25mg/ml) Ampicillin) and cultured at 37°C overnight. Numerous (white) recombinants were obtained and colonies were amplified by adding four colonies to 5ml TX medium with 50μl Ampicillin (25mg/ml) and shaking overnight at 37°C in a waterbath. A plasmid preparation of the pGEM blue was performed according to the method of Davis et al. (1986).

Orientation of the insert was such that in the PGEM-3Z vector the Kpn-1 end of the insert orientates 3' to the Pst-1 site. To obtain an anti-sense RNA transcript the PGEM-3Z is cut with Hind III and the T7 promoter is used for the transcription reaction.

Dot blots of the plasmid preparation (1μl) probed with nick translated CFLV-108 probe, confirmed that the PGEM plasmid preparation contained an insert of the human beta-2 receptor gene.

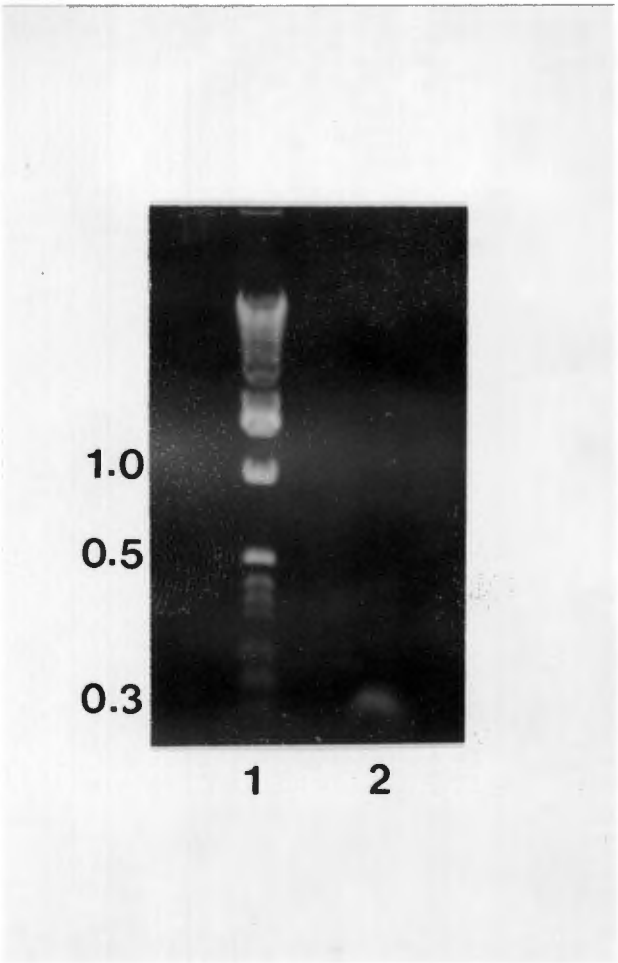


Figure 32. Agarose gel of 326bp KpnI-Pst-1 human beta-2 receptor probe (lane 2) purified by electroelution. 1 kb ladder (lane 1).

Cold 326 bp RNA transcripts were generated using the Promega Gemini T^m system in a 100μl reaction volume with reagents added in the following order: 39μl DEPC treated H_2O, 20μl 5x transcription buffer, 10μl 10mM DTT, 4μl RNasin (25 units/μl), 20μl (2.5mM each of ATP, CTP, UTP and GTP made by mixing 5μl of 10mM stock ATP, CTP, UTP and GTP together), 5μl of DNA (~2μg in T.E. buffer, linearised with Hind III) and 2μl T7 RNA polymerase. After a 2 hour incubation at 37°C, 2μl of RNase free DNase-1 was added, incubated for a further 20 min at 37°C, after which the RNA was extracted with an equal volume of phenol and chloroform, the riboprobe was precipitated with a 1:10 volume of 3M sodium acetate pH 6 and 2.5 volumes of ice cold 95% ethanol. The RNA transcript was visualised directly on agarose gels and confirmed to be 326bp.

A RNA probe of high specific activity was synthesized in a reaction volume of only 20μl, containing 5μl (10mCi/ml) of [α-^{32}P] CTP (according to the Promega manufacturer's instructions). Transcription reactions resulted in specific activity of the riboprobe of 0.9×10^8 - 5×10^9 cpm/μg template.

Specificity of the anti-sense 326bp beta-2 receptor riboprobe for the 2.6kb sense transcript of the beta-2 receptor RNA was confirmed (Fig. 27). While I was still at the NIH, this probe was given to Dr. Jean Paul Giaciobino of the University of Geneva, Department of Physiology, for studies on adipocyte RNA induction (4.2.6) and was subsequently also sent to the Brompton Chest Hospital (London), personal communication (Dr. P. Barnes) for *in situ* hybridization studies of the human lung beta-2 receptor (which were presented at the 8th Congress of SEP European Society of Pneumology and European Paediatric Society, September 1989 at the University of Freiburg (FRG)).

4.2.6 Identification of the beta 1 receptor mRNA using the 326bp probe.

The 326bp fragment which I cloned into pGEM-3 is derived from a highly conserved second transmembrane region of the beta-2 adrenergic receptor gene. Comparison of this gene sequence of the human beta-2 adrenergic receptor with the published gene sequence (Yarden et al. 1986) of the turkey erythrocyte beta adrenergic receptor, (beta-1 receptor) using Gen Data base, revealed a 69.6% homology, suggesting that this 326bp probe could be used to identify the beta-1 receptor gene. This possibility was particularly interesting, since previous attempts to identify the human beta-1 receptor gene with human beta-2 adrenergic receptor probes had not been successful (Frielle et al. 1987). I reasoned that if an RNA species could be identified which differed in size from the human beta-2 receptor mRNA which I identified in 4.2.3, using this probe, the 326bp probe could be successful in screening an adipocyte cDNA library to isolate the human beta-1 gene, since adipose cells were believed to express the beta-1 receptor exclusively.

Briefly, 50μg Poly(A)$^+$ RNA was prepared from rat fat epididymal fat pad (beta-1 receptor subtype specific), rat heart (beta-1 receptor subtype predominant) from normal and from hypothyroid rats (a gift from Dr. J.P. Giaccobino, University of Geneva), northern blotted on to nitrocellulose, and probed with a random primer labelled 326bp probe. A mRNA species of 2.5kb (Fig. 33) was detected in the rat heart and rat adipose tissue Poly(A)$^+$ RNA. This is a distinctly larger species

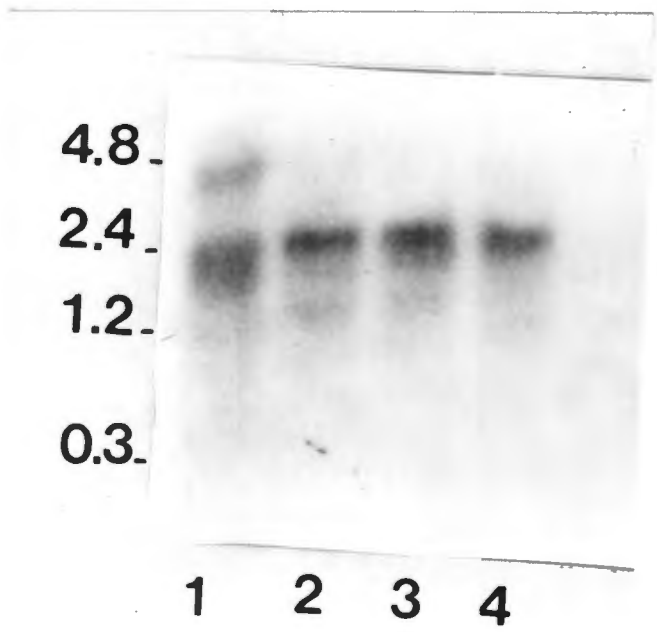


Figure 33. Autoradiograph of Northern blots of poly(A)$^+$ RNA from normothyroid rat heart, (50μg)(lane 2), hypothyroid rat heart (50μg)(lane 3), brown fat (39μg)(lane 4) probed with 326bp random primer labelled probe identifying a 2.5kb RNA species in each. BRL RNA ladder (lane 1) demonstrated non-specific labelling to 2.4kb and 4.8kb markers.

than the 2.2kb beta-2 receptor mRNA species which I had detected using the CLFV-108 (beta-2 receptor) probe in the W1-38 cells. A 2.5kb mRNA species was confirmed in rat heart (50μg Poly A) when a riboprobe of the 326bp construct was used (Fig. 34). At about the same time that these experiments were performed, the human beta-1 receptor gene was cloned and sequenced by Frielle et al. (1987) using the human genomic clone G21. These workers also found a mRNA of 2.5kb for the B-1 receptor in Northern blot hybridization studies of rat mRNA.

4.2.7 Summary of the beta-2 receptor RNA studies

At the time when these studies were commenced nothing was known about the human mRNA species for the beta-2 receptor gene. I assumed that the mRNA species would be reasonably abundant in organs expressing beta-2 receptors and thus guinea pig lung tissues were selected for the initial studies. Once the preliminary experiments were conducted to optimise the yield and quality of RNA isolation, I attempted to identify the mRNA species in the guinea pig lung using Northern blotting. Repeated failure to identify beta-2 receptor mRNA in Northern blots of as much as 50μg Poly(A)$^+$ RNA suggested that the RNA species was rare, readily degradable, or had a short half life, possibly related to the fact that the beta-2 receptor gene has no introns. These results also indicated that further studies on induction of beta-2 receptors in the developing guinea pig lung at the level of gene transcription were unlikely to succeed using the technique of Northern blotting of Poly(A)$^+$ RNA. Rarity of the mRNA

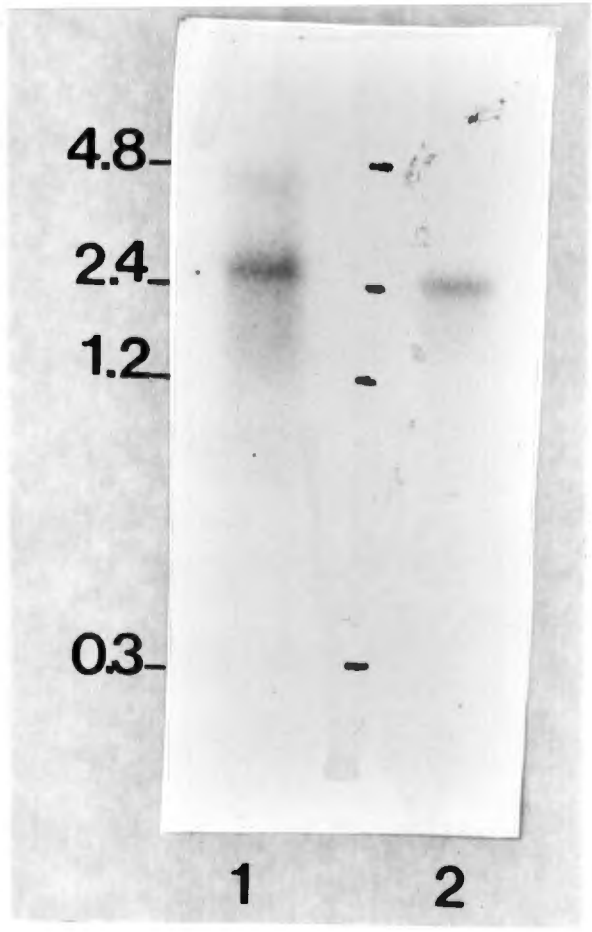


Figure 34. Autoradiograph of a Northern blot of poly(A)$^+$ RNA from rat heart (50μg)(lane 1) probed with the 326bp riboprobe identifying the 2.5kb RNA species. Non-specific labelling of 2.4kb marker on BRL RNA ladder shown in lane 2.

species in placental tissue was alluded to by Dixon (1986) and Kobilka (1987).

In spite of the initial difficulties with the guinea pig lungs, I did attempt a second gestational study while I was at the NIH. I prepared foetal lungs from pregnant guinea pigs at 16 gestational time points from 48 days to 66 days gestation. (Timed matings were performed by Hazelton Research Laboratories, Denver). Animals were killed by intraperitoneal injection of pentobarbitone (Somnifer, Richmond Veterinary Supply Company, Virginia) and foetal lungs (total 73 foetuses) were snap frozen and stored at -70°C before RNA (total and Poly(A)$^+$ RNA was isolated using the method of Chirgwin et al. (1979). Northern blots of up to 50μg of Poly(A)$^+$ RNA from the term foetuses did not identify a positive mRNA species when probed with either the cDNA probes or with the beta-2 receptor riboprobes I had constructed, confirming the difficulties I had encountered previously with the guinea pig lung tissues and thus further studies on the more immature lungs were abandoned (for both technical and economic reasons).

In view of the apparent rarity of the mRNA species, a 2.6kb riboprobe for the beta-2 receptor gene was then constructed and sense and anti-sense transcripts were used as positive controls in the subsequent Northern blots. The riboprobe was no more successful in identifying the mRNA species in guinea pig lung tissues than the cDNA probes on the Northern blots.

Positive identification of a 2.3 kb beta-2 adrenergic mRNA species in Wl-38 cells (using Northern blots) with the CLFU 108 cDNA probe, was followed by studies of the induction of beta-2 receptor mRNA by steroids in Wl-38 and Va4 cells at the level of gene transcription.

Preliminary studies using slot blots showed a clear increase in hybridization signals in the RNA from dexamethazone probed cells which were not seen in controls. However, these increases were difficult to demonstrate convincingly on Northern blots. Use of the 326bp probe (cDNA or riboprobe) did not improve the detection of the mRNA species on Northern blots, but it did identify a different, 2.5kb mRNA species in tissues selectively expressing beta-1 receptors which was compatible with the beta-1 receptor RNA species. Duman et al. (1989) were more successful in detecting changes in beta receptor RNA with development and has shown, using Northern blots, that in the post-natal rat brain, the level of beta-1 and beta-2 adrenergic receptor mRNA followed a pattern which parallelled that of receptor binding sites.

The studies on Wl-38 cells and the studies of Collins et al. (1988) demonstrate that beta-2 receptors can be transcriptionally regulated by glucocorticoids leading to a rapid rise in the steady state of beta-2 adrenergic receptor mRNA. These findings have implications for a possible mechanism of the beneficial effect observed when patients with asthma are treated with steroids, implying that one of the effects of steroid therapy is to induce beta-2 receptor expression at the level of gene transcription.

The possibility that disturbances in transcriptional regulation of beta-2 receptors may occur in the airways of allergic asthmatic subjects has not previously been investigated, but may be an interesting area for future studies.

4.3 *In vitro* models of dysfunctional beta-2 receptors: Studies of the beta-2 adrenergic receptor ligand binding domain using mutant beta-2 adrenergic receptors.

Cloning and sequence analysis of the beta-2 adrenergic receptor has shown that this receptor belongs to a family of transmembrane proteins which are coupled to guanine regulatory proteins (G proteins). This family includes rhodopsin, the visual colour opsins, the muscarinic receptors, the $alpha_2$ adrenergic receptor and the serotonin 1c receptor, all of which have seven hydrophobic transmembrane regions. Comparison of the gene sequences and deduced protein sequences of several receptors which utilise similar transduction mechanisms, facilitates the identification of common residues which participate in, and are necessary for, coupling to the G proteins and, furthermore, facilitate the identification of the residues which confer the selective structural or conformational specificities to the molecule which are complimentary to their different respective ligands.

The theoretical possibility that the beta-2 receptor gene sequence may be subject to spontaneous point mutations *in vivo*, leading to varying degrees of receptor dysfunction which may result in, or aggravate

clinical disease processes is worth investigating, since point mutations of other human proteins leading to clinical disease are well described (eg. in cystic fibrosis). Genetic differences, however, rarely occur within the coding region of the gene, and are more commonly attributed to mutations in regulatory sequences of the gene.

Polymorphic differences in the DNA sequences of the beta-2 receptor were first reported by Lentes et al. (1988) who described a restriction fragment length polymorphism of the human beta-2 receptor (using the enzyme Ban 1) in normal North American subjects.

These studies were remarkable in that no polymorphism was found when 47 other restriction enzymes were used, suggesting that variations in the beta-2 adrenergic receptor gene sequence are uncommon. This is not surprising if one notes that the beta-2 receptor gene structure is highly conserved in nature (Venter et al. 1988) and it is likely that mutations in critical ligand binding domains of the receptor may be lethal.

Once the beta-2 receptor gene was cloned and sequenced, an expression vector was constructed by Chung et al. (1987, 1988) and stable transfection of the wild type receptor in B82 cells was achieved.

The mutant beta-2 receptors which I studied and describe in this section were constructed by Dr. Fuzon Chung at the N.I.H., Bethesda. and I assisted him with and performed several of the mutant gene transfections of the B82 cells. My own specific project, however, was

to perform the initial biochemical characterization (binding assays) on the first nine (of a total of 20) mutants in order to identify mutants which would be worth studying in more detail by other members of the group and myself after the initial characterization. Data I obtained for each of these mutants is presented in 4.3.2. I then proceeded to study in more detail two of the mutants, (Asp 79 as part of a team and the glycosylation mutant Asn 6 on my own) while the other six mutants were allocated to other members of Dr. Craig Venter's group for further characterization.

While these mutants were constructed primarily to identify the critical ligand binding and coupling residues, they provide, at the same time genetically engineered models of mutations which if occurring spontaneously could theoretically result in dysfunctional beta-2 receptors in human disease.

4.4.1 Mutant construction and transfection

A total of nine point mutations of the human beta-2 receptor amino acid sequence were constructed after studying the model proposed by Kobilka et al. (1987) which proposed an N-terminal extracellular tail of 34 amino acids, seven transmembrane hydrophobic domains and an extended C-terminal intracytoplasmic domain of 85 amino acids. The sites of the mutant beta-2 receptors which I studied are shown in Fig. 35 (sequence model adapted from O'Dowd et al. 1989)

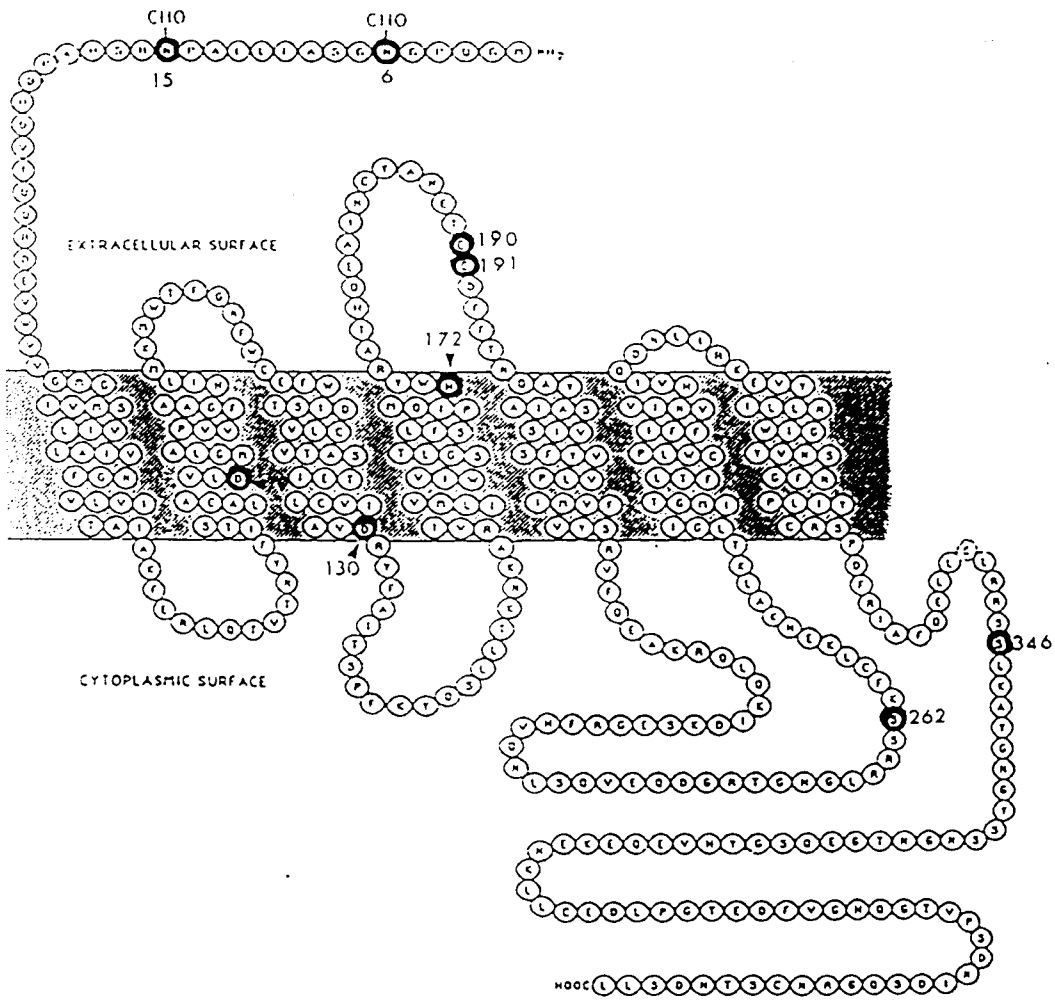


Figure 35. Sites of point mutations performed on 9 mutant human beta-2 adrenergic receptors studied (4.4.1). Sequence model adapted from O'Dowd et al (1989).

Point mutations were constructed by oligonucleotide directed mutagenesis, according to the method of Kunkel et al. (1987), using the Biorad Muta-gene^{Tm} *in vitro* mutagenesis kit (Cat. 170-3571). Authenticity of the mutations was checked and confirmed by dideoxy sequencing of the mutated genes, with the assistance of Dr. Dianne Wang.

For receptor expression, the mutated coding region of the human beta-2 receptor gene originally derived from the wild type genomic clone LCV-517 (base pairs - 6 to + 1267) was cloned into the Mlu and Eco RV sites in the polylinker region of pMSV neo vector (Chung et al. 1988) and we then transfected the genes into B82 cells and selected clones with successful transfections using Geneticin (G418-Sulphate Gibco Cat 860-1811) in the same way as I transfected the wild type beta-2 receptor gene into B82 cells when I returned to Cape Town (described in detail in Chapter 6.2.1).

The amino acid substitutions and gene sequence changes in the nine mutant receptors studied are listed in Table 12.

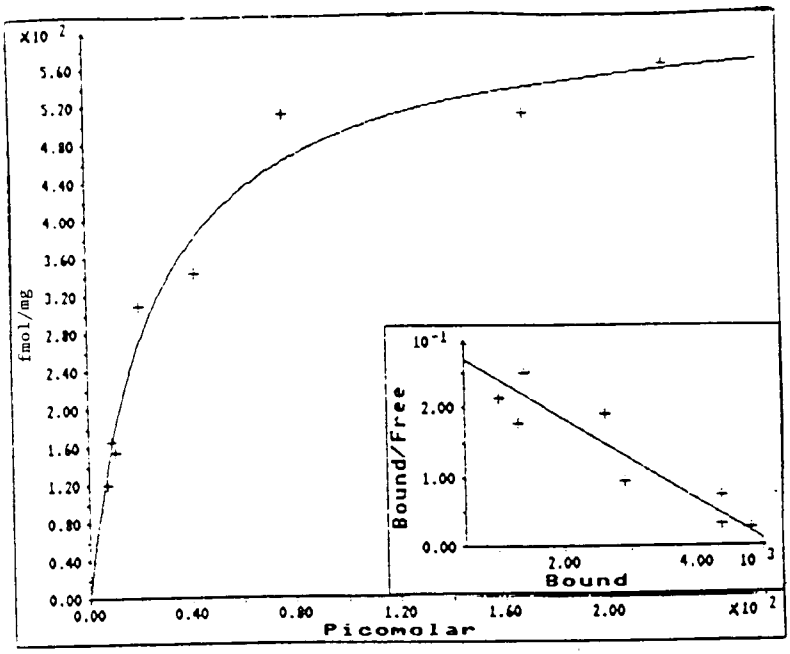
Prior to commencing the studies with the mutants I performed saturation binding curves and Scatchard plots on three other clones (Clone NE, 82A, 82C) of wild type receptors which we had transfected into B82 cells (Fig. 36), (these were different clones to those reported by Fraser et al. (1987)) to obtain reference binding data with which to compare the nine mutants. Binding data for the three wild type receptors and the mutants have been analysed using the non linear

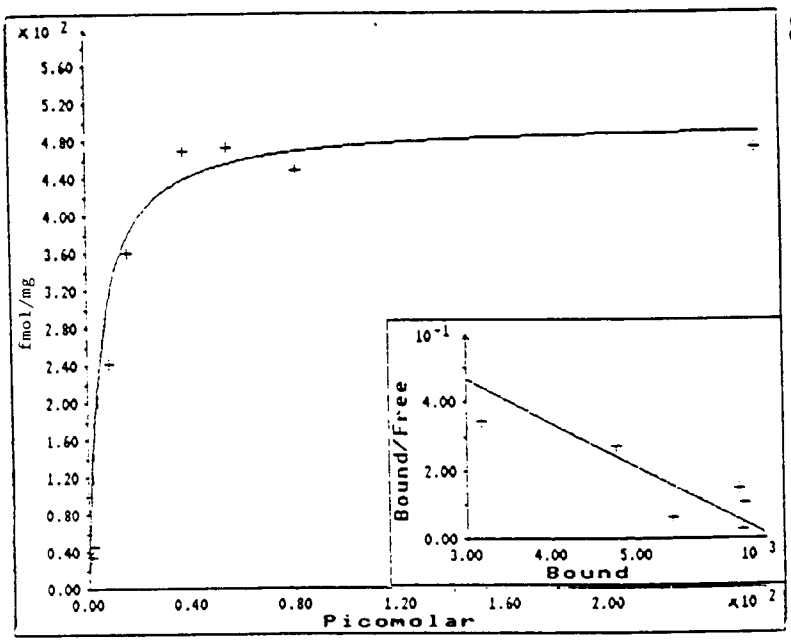
Figure 36. Saturation curves and Scatchard plots of [^{125}I]-CYP binding to 3 clones of B82 fibroblasts transfected with the wild type human beta-2 adrenergic receptor gene.

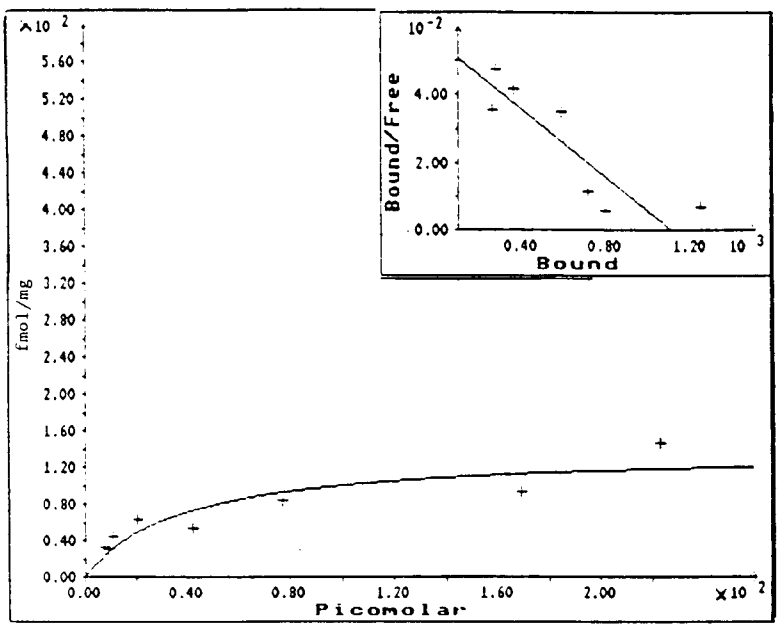
Clone NE Bmax 626 fmol/mg kD 26.9

Clone 82c Bmax 490 fmol/mg kD 25.8

Clone 82A Bmax 135 fmol/mg kD 34.8







125I-CYP Specific Binding

125I-CYP

Figure 36.

iterative regression data analysis programme ENZFITTER (IBM, PC) by Leatherbarrow, Department of Chemistry, Imperial College of Science, London.

While the level of expression in the three wild type human beta-2 receptor clones was fairly variable, the affinity of the wild type receptors for [^{125}I]-CYP was similar (Table 13).

Table 12

Site	Amino acid change	Gene sequence change	Region of secondary structure
Asn 6	Asn Gln	AAC CAG	N Terminal
Asn 15	Asn Gln	AAT CAG	N Terminal
Asp 79	Asp Asn	GAT AAT	2nd Transmembrane
Asp 130	Asp Asn	GAT AAT	3rd Transmembrane
His 172	His Asp	CAC GAC	4th Transmembrane
Cys 191	Cys Ser	TGC AGC	between 4 and 5
Cys 190-Cys191	Cys Ser	TGC AGC	between 4 and 5
Ser 262	Ser Ala	TCC GCC	between 5 and 6
Ser 346	Ser Ala	TCT GCC	C Terminal

Table 13

Wild Type Clone designation	B Max fmol/mg	Kd (pM)
NE	626.41	26.97
82C	490.33	25.78
82A	135.45	34.76

4.3.2 Partial characterization of 9 beta-2 adrenergic receptor mutants

B82 cells, transfected with 9 mutant and 3 wild type beta receptors were cultured to semi-confluency in 25 cm culture dishes (in 40% Dulbecco's medium, 40% F-12 medium and 10% fetal calf serum, supplemented with glucose, glutamine, non essential amino acids, Penicillin and Streptomycin)(see appendix E) in the presence of Geneticin (G418 sulphate, final concentration 500μg/ml) and then pulsed overnight with 10^{-6}M dexamethazone (Sigma) to induce beta adrenergic receptor expression.

Cells were lysed at 4°C using hypotonic 5mM sodium phosphate 2mM $MgSO_4$ pH 7.4 for 30 min, scraped off the dishes and homogenised with a glass

homogeniser and then centrifuged at 1000g at 4°C for 10 mins to remove cell nuclei and other cell debris. The supernatant was then centrifuged at 40 000g for 40 mins at 4°C to pellet the cell membrane fraction. Membranes were resuspended in 100μl aliquots in 20mM sodium phosphate 2mM $MgSO_4$ at an approximate concentration of 4mg/ml and stored at -70°C or in liquid nitrogen. For these studies membrane protein was assayed using the fluorescamine assay with bovine serum albumin as a standard (Bohler, 1973).

Saturation of [^{125}I]-CYP binding to membrane preparations of the mutant beta-2 receptors and Scatchard plots of the binding data are shown in Figs. 37-39. Binding data was obtained using ENZFITTER.

The binding studies of the nine mutants were most informative. Certain mutants appeared to have normal antagonist binding (Asn 6, Asp 79, Asp 130, Ser 262, Ser 346) while a significant reduction of [^{125}I]-CYP binding affinity data was observed with the mutant Cys 191. No specific binding was obtained with Asn 15 and only non-specific low affinity binding was observed with mutant His 172 and Cys 190-191. Maximal binding (Bmax) and [^{125}I]-CYP dissociation constants obtained from the saturation curves and Scatchard plots of these nine mutants are summarised in the following table:

Table 14

Mutant Studied	Amino Acid change	Gene Sequence change	[^{125}I]-CYP binding Bmax fmol/mg	Kd(pM)
Asn 6	Asn-Gln	AAC-CAG	276	5.94
Asn 15	Asn-Gln	AAT-CAG	NSB	-
Asp 79	Asp-Asn	GAT-AAT	227	19.06
Asp 130	Asp-Asn	GAT-AAT	20	17.50
His 172	His-Asp	CAC-CAG	NSB	-
Cys 191	Cys-Ser	TGC-AGC	194	258.52
Cys 190-191	Cys-Ser	TGC-AGC	NSB	-
Ser 262	Ser-Ala	TCC-GCC	184	10.3
Ser 346	Ser-Ala	TCT-GCC	424	10.54

(NSB = No specific binding)

(Cys 190-191 was a double mutant)

Expressed mutants which displayed [^{125}I]-CYP binding were chosen for detailed functional characterisation and it was decided that we would characterise Asp 79 mutant as a group, Dr. Claire Fraser the Asp 130 and the two cysteine mutants, Dr. Chung was assigned the mutant for Ser 262, and I would study the Asn 6 mutant. (The serine mutant 346 was not part of the initial characterization performed at the NIH but I transfected the mutant gene into L cells for the first time on my return to Cape Town, using the protocol I have discussed in Chapter 6, after receiving the plasmid containing the mutant from Dr. Chung.)

Unfortunately, Dr. Chung could not proceed with further characterization of the 262 serine mutant since he left the NIH to take up a commercial appointment.

4.3.3 Mutants displaying absent or altered [^{125}I]-CYP binding:

The mutants which displayed no apparent [^{125}I]-CYP specific binding, i.e. His 172, and Asn 15, or very low affinity binding (e.g. Cys 190-191) presented the possibilities that either the mutant protein was not expressed at all and the cells retained the geneticin resistant gene, or that the mutation in fact conferred important effects on the conformation of the expressed receptor, which impaired antagonist ligand binding. The laboratory in which I was working at the N.I.H. did not, at that time, have available good polyclonal or monoclonal antibodies to the beta-2 adrenergic receptor (Venter and Fraser had lost their anti-beta receptor antibody secreting clones during a move), thus it was not possible, at that time, to be sure that immunoreactive receptor was indeed expressed in the mutants in which no specific [^{125}I]-CYP binding was detected. I did attempt to obtain anti-beta-2 receptor antibodies from two other laboratories but was unsuccessful. Mutant sequences were checked by Dr. Diane Wang by dideoxynucleotide sequencing.

The Cys 191 mutant (Fig. 37) was of great interest because although L(-) propranalol displacable [^{125}I]-CYP binding was present, the binding was of low affinity (Kd 258.52pM) suggesting that the cysteine residues in the extracellular domain of the beta-2 receptor

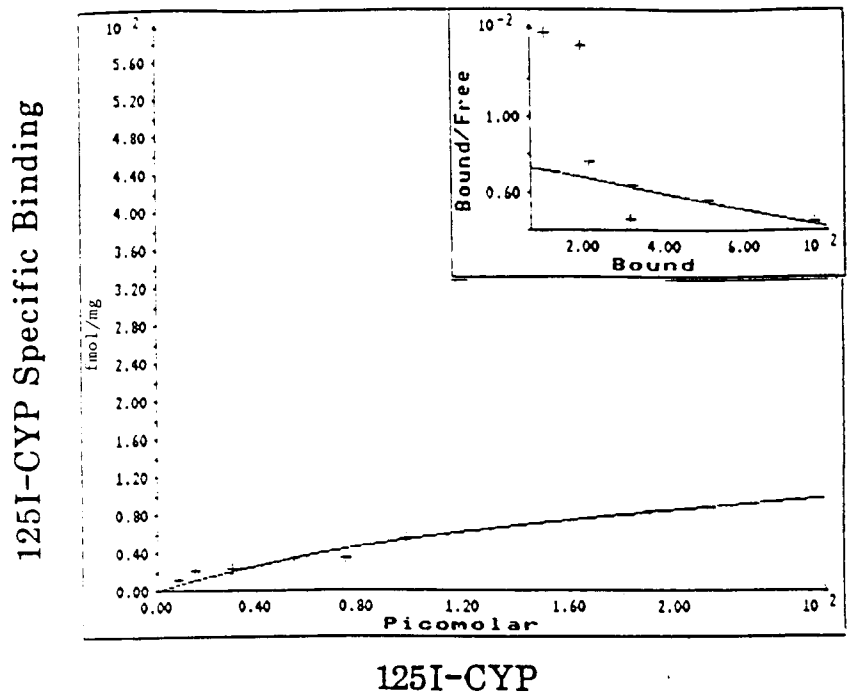


Figure 37. Saturation curve and Scatchard plot of [^{125}I]-CYP binding to Cys 191 (Cys-Ser) mutant of the human beta-2 adrenergic receptor in membranes of B82 cells. Kd = 258.52pM Bmax 194fmol/mg.

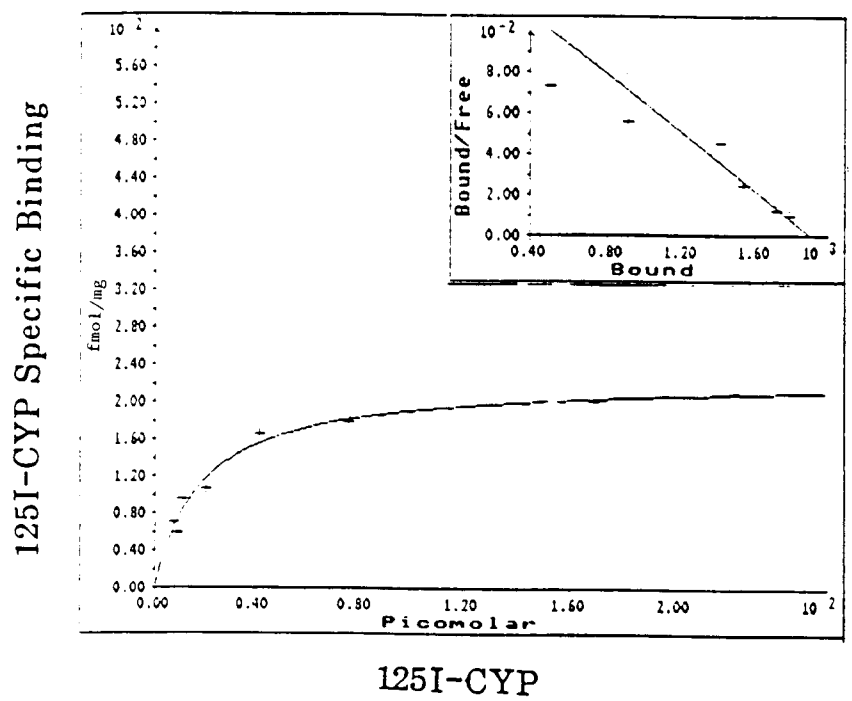


Figure 38. Saturation curve and Scatchard plot of [^{125}I]-CYP binding to Asp 79 (Asp-Asn) mutant of the human beta-2 adrenergic receptor. Bmax 227fmol/mg Kd 19.1 pM.

may either be involved directly in ligand binding, or may confer important conformational specificities necessary for normal ligand binding.

Subsequent studies (by Fraser 1989) involving re-transfections with the Cys 190-191 and Cys 191 mutants on which I had conducted the initial studies, and the construction of three more cysteine mutants, showed that replacement of cysteine residues with serine at sites 77 and 327 (in the second and seventh transmembrane-spanning domains) had no effect on ligand binding or the ability of the receptor to mediate isoproterenol stimulation of adenylate cyclase. Substitution of cysteine 285 resulted in attenuated adenylate cyclase activation but mutation of residues 190 and 191 in the third extracellular loop had qualitatively similar effects, with a loss of affinity for ligand binding and a reduction of isoproterenol stimulation of adenylate cyclase. The replacement of both cysteine 190 and 191 with serine had a marked reduction in ligand affinity and functional activity of the receptor.

The His 172 mutant displayed a somewhat bizarre [^{125}I]-CYP saturation curves with no specific binding and has not been studied further.

4.3.4 Aspartate mutants

A saturation curve and Scatchard plot of [^{125}I]-CYP binding to Aspartate 79 mutant is shown in Fig. 38. Binding studies showed that replacement of the Aspartate 130 and Aspartate 79 with Asparagine

residues had no appreciable effect on antagonist [^{125}I]-CYP binding to the receptor. The Asp 130 mutant displayed normal binding affinity (Kd 17.5 pM) but there was a low level of expression (Bmax 20fmol/mg), and has not yet, to my knowledge, been characterised further. The aspartate residue at position 79 is highly conserved in all beta adrenergic, α-adrenergic muscarinic receptors and opsin proteins. (See Appendix G).

The aspartate residue at position 79 in highly conserved in all beta adrenergic,

Published results of our further characterization of the Asp 79 mutant are discussed in detail in the Journal of Biochemistry Vol 263, March 1988. A reprint of this publication is included in Appendix G. Briefly, we found that substitution of aspartate 79 with asparagine produced a mutant receptor that displayed expected affinity and stereoselectivity for antagonists, but a 40-, 140- and 240-fold reduction in its affinity for isoproterenol, epinephrine and norepinephrine, respectively. This mutant also showed a marked impairment in cyclic AMP production when compared with wild type cells. These studies confirmed and extended the studies of Strader et al. (1987) who substituted Asp 79 with an alanine in the hamster lung β-2 adrenergic receptor and found a 10-fold loss in receptor affinity for agonists. Studies with these mutants demonstrated that a negatively charged aspartate was essential for normal agonist binding to the receptor, and also suggested that an aspartate residue at amino acid 79 may be necessary to facilitate coupling of the receptor to the G proteins.

Studies by Dixon et al. (1987), in which the importance of sequential deletions in the gene for the hamster beta adrenergic receptor encompassing the protein coding region was studied using transient expression in COS-7 cells, suggested that most of the hydrophilic

regions appear to be dispensable for ligand binding. Deletion mutants encompassing amino acids 70-96 showed no ligand binding and failed to be translated into a detectable 67kDa protein. Other mutant receptors in which hydrophobic helices were deleted, including deletions of amino acids 33-105, and 64-140 similarly did not express immunoreactive protein. In a subsequent communication, Strader et al (1989) found that substitution of Asp 113 in helix 3 affected the binding of both agonists and antagonists to the receptor, but that coupling to the adenyl cyclase system was not affected, and suggested that the amino group of catecholamine ligands form an ion pair with the carboxylate side chain of Asp 113. It is not known to what extent ion pairing also occurs between the Asp 79 and the amino group of the ligand. Support for Strader's hypothesis carries from the observation that an Aspartate residue in helix 3 is conserved among all of the G protein linked receptors that bind amine ligands, including all the subtypes of α- and β-adrenergic, muscarinic and serotonergic receptors, but not those receptors whose ligands are not protonated amines, such as the substance K and angiotensin receptors, or the opsins.

4.3.5 Glycosylation mutants of the human beta-2 adrenergic receptor.

Point mutations of the beta-2 adrenergic receptor at the residues Asn 6 and Asn 15 on the extracellular N-terminal loop of the protein, were constructed by Dr. F. Chung (see Table 12) and I performed the transfections and the partial characterization of these mutants. Repeat transfections with the Asn 15 (G54) mutant at no stage showed propranalol displacable [^{125}I]-CYP binding, and this was later

attributed to the fact that this mutant had inadvertently been severely truncated (Fraser, personal communication), although a "proximal" sequence of the mutated gene confirmed that the Asn had been correctly mutated, at the amino acid residue 15.

B82 mouse cells were successfully transfected with mutant Asp 6 beta-2 receptor DNA and 12 geneticin G418 resistant clones were selected, amplified in 24 well plates and screened for L(-) propranalol displacable [^{125}I]-CYP binding at a single concentration (200pM). The highest expressing clone (G53) was chosen for further binding studies.

The Asp 6 clone, (designated G53) showed saturable, stereospecific binding of [^{125}I]-CYP, Bmax 276fmol/mg Kd 5.94pM, which was highly reproducible and a representative saturation curve and Scatchard plot is shown in Fig. 39.

Photo affinity labelling using [^{125}I]-CYP-diazirine (as described in 2.2.11) of the clone G53 (Asn\Gln mutant) showed propranalol displacable photolabelling between MW 62 000 and 94 000 daltons (Fig.40). [^{125}I]-CYP-diazirine labelled both wild type transfected and Asn 6 mutant receptors, and the size of the molecules labelled suggested that the Asn 6 mutant receptor was still glycosylated.

Direct comparison of [^{125}I]-CYP diazirine photolabelling of 1mg/lane of membrane proteins from non-transfected B82 cells, the beta-2 receptor transfected wild type receptors (200fmol/mg) and Asn 6 mutant (200fmol/mg) receptors shows an apparent reduction in the intensity

and size of the photolabelled receptors in the Asn mutant suggesting that the mutant receptor was partially glycosylated and of apparent lower molecular weight. Since this finding also could have resulted from an "error" in the mutation, the mutated sequence of the mutant Asn 6 was checked again and confirmed by Dr. D. Wang. Dideoxy nucleotide sequencing confirmed that the mutated sequence had in fact changed from AAC to CAG at residue 6.

In order to confirm that Asn 6 was partially glycosylated, membranes were prepared from cells grown for 24 hours in 0.1μg/ml Tunicamycin. In several independent experiments, photolabelling of these membranes with [^{125}I]-CYP-diazirine consistently showed an unimpressive decrease in the intensity of the labelling and did not result in the clear appearance of a lower molecular weight species as expected from the studies performed by George et al. (1986) of S49 mouse lymphoma cells. These data suggested that the glycosylation was not inhibitable by tunicamycin at the concentration studied, or that glycosylation due to golgi alpha-1,2 mannosidase II may also be important. I have not done further studies with other inhibitors of glycosylation. Boege et al. (1988) have commented on different cellular sensitivities to tunicamycin treatment in a study of the function of non-glycosylated beta-2 adrenergic receptors prepared from tunicamycin (150ng/ml) treated A431 cells which had been reconstituted in lipid vesicles, and suggested that protein bound carbohydrate was indeed required for β adrenoceptor function in normal signal transduction. In particular, coupling with Gs protein was impaired with the unglycosylated receptor. These findings were in contrast to those of Benovic et al (1987) who found

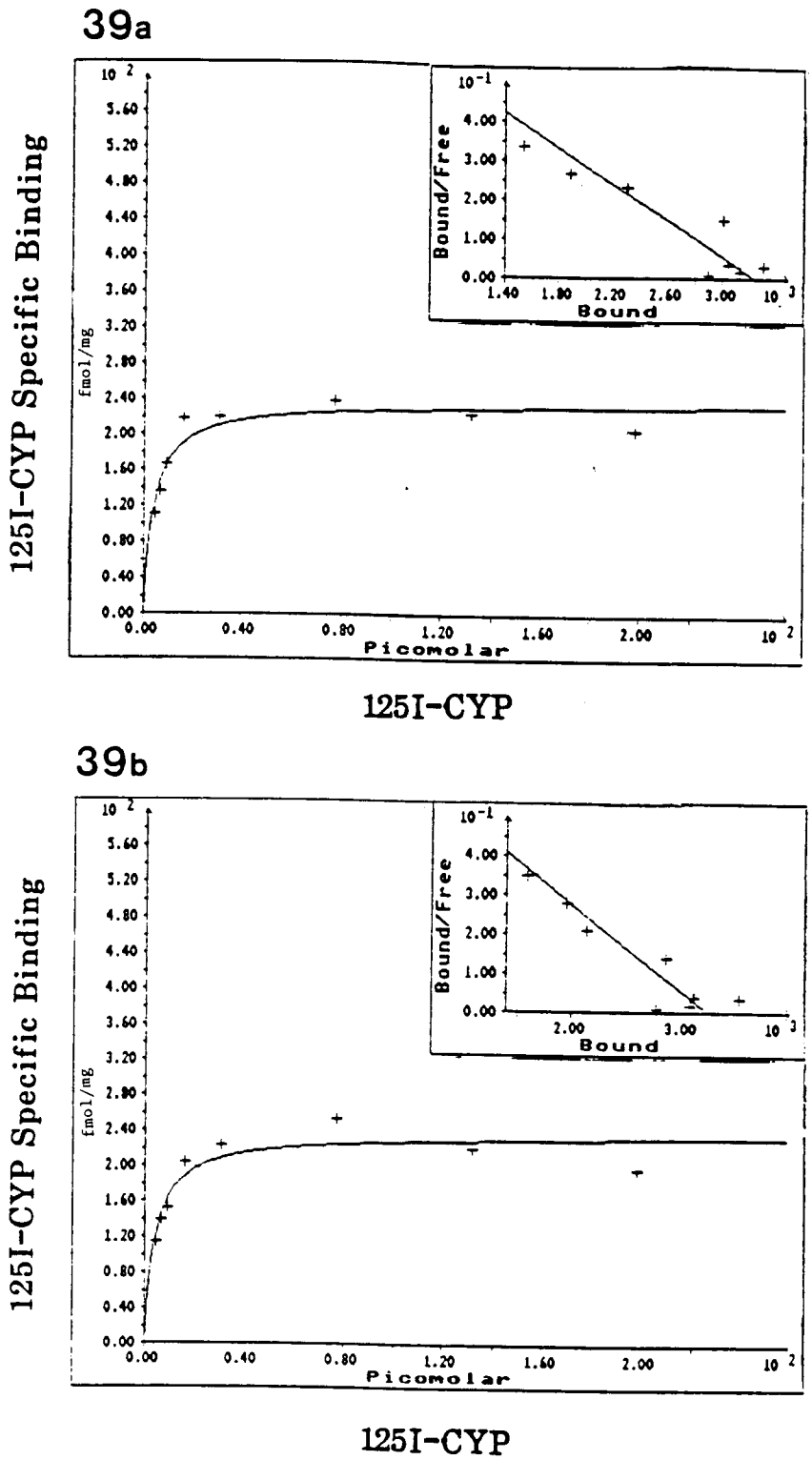


Figure 39. Saturation curves and Scatchard plots of [^{125}I]-CYP binding to Asp6 (Asn-Gln) mutant of the human beta-2 adrenergic receptor cultured in the absence (39a) or presence (39b) of Tunicamycin C.

Asn mutant Bmax 237 fmol/mg Kd 4.1pM

Asn mutant (with Tunicamycin) Bmax 236 fmol/mg Kd 4.16pM

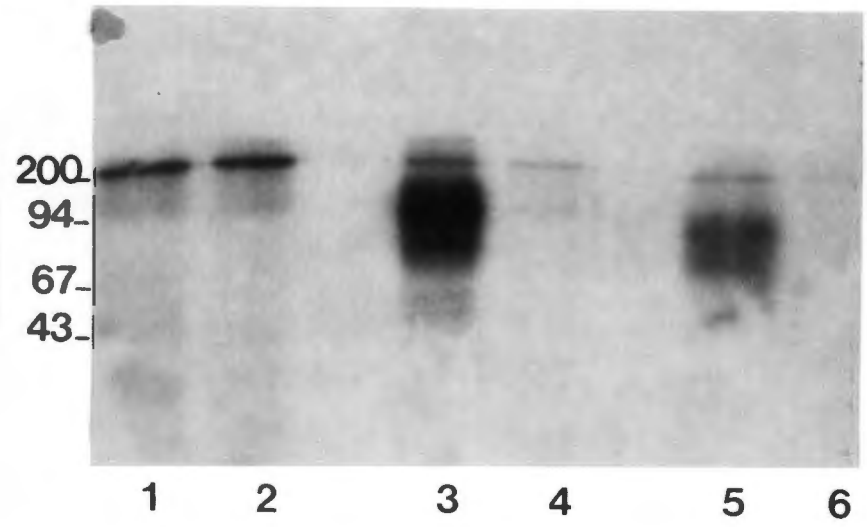


Figure 40. Autoradiographs of photoaffinity labelling of membranes prepared from untransfected B82 cells (lanes 1 & 2). B82 cells transfected with the wild type beta-2 receptor gene (lanes 3 & 4) and B82 cells transfected with the Asn 6 mutant gene (lanes 5 & 6). Labelling in lanes 2, 4 and 6 was performed in the presence of 10^{-5}M (L)propranalol.

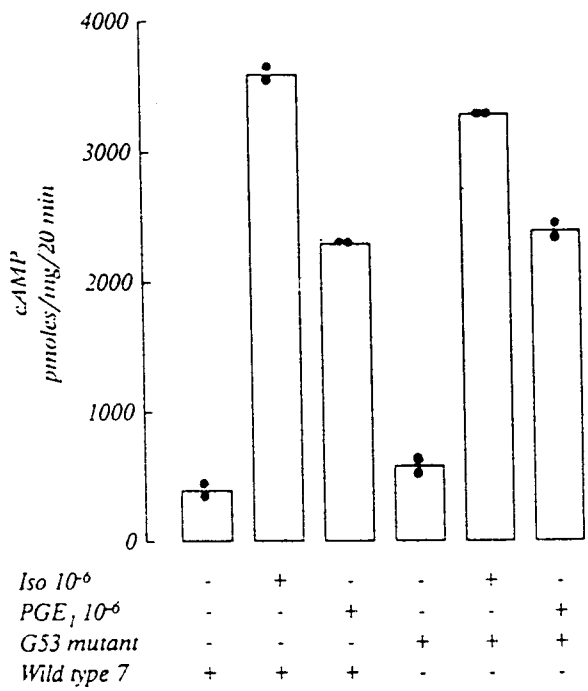


Figure 41. 10^{-6}M isoproterenol and 10^{-6}M prostaglandin E_1 stimulation of cAMP production in B82 cells transfected with wild type human beta-2 receptor and in B82 cells transfected with Asn 6 (G53) mutant human beta-2 receptor. Bar values represent the mean of duplicate assays (.).

unchanged coupling of non-glycosylated purified hamster beta-2 adrenoceptor with Gs. Benovic attributed the discrepancy to the reconstitution system employed by Boege.

In order to investigate the function of the transfected Asn 6 mutated beta-2 receptors, I performed adenylate cyclase assays (Amersham) on the transfected cells. A brisk cAMP response to 10^{-6}M isoproterenol was obtained (Fig. 41), comparable with that of the wild type beta-2 receptors. (The protocol for the adenylate cyclase assays is described in detail in 6.2.3).

These studies have shown that mutation of the Asn-6 residue did not significantly alter agonist activation of adenylate cyclase or expression of the receptor in the cell membrane of transfected cells, but further studies are required.

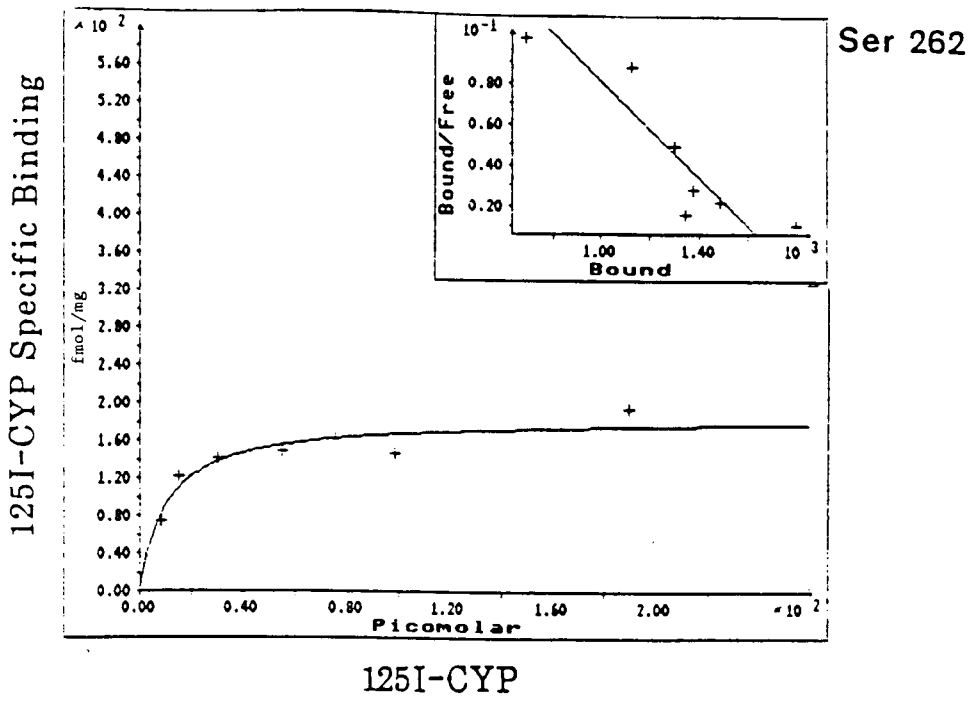
The role of the carbohydrate moiety in structure and function may differ from one receptor to another, as in the case of the turkey Beta-1 adrenoceptor and the mammalian beta-2 adrenoceptor (Yarden et al. 1986, Dixon et al. 1986). It is also evident from the literature that while glycosylation appears to be critical for translocation to the cell membrane in certain receptors, e.g. Complement receptor CR1 (Lublin et al. 1986) incomplete glycosylation was without functional effect for other receptors e.g. insulin-like growth factor receptor (Duronio et al. 1986).

4.3.6 Serine mutants of the beta-2 adrenergic receptor

Binding studies with [^{125}I]-CYP have demonstrated normal affinity of binding for these mutants. I have achieved a level of expression for the Ser 346 - Ala mutant which is approximately double that achieved for the Ser 262-Ala mutant. Saturation curves and Scatchard plots are shown in Fig. 42. Both mutants displayed good 10^{-6}M isoproterenol stimulated cAMP production (cAMP production in Mutant 346 is shown in Fig. 43) but I have not studied the affinity of agonists in further detail. In particular, the effects of these two mutations on phosphorylation of the beta-2 receptor will be important to study in the future. Dixon (in press) has found that amino acid substitutions at Ser 204 and Ser 207 in helix 5 and Ser 319 in helix 7 affected agonist but not antagonist binding to the receptor.

4.3.7 Subsequent studies on other beta-2 adrenergic receptor mutants

Since the initial studies of beta-2 receptor mutants, discussed above, several important mutational studies have been published by Strader et al. (1989) O'Dowd et al. (1989), Kobilka et al. (1988) and others. All of these studies, while providing vital information, have also illustrated some of the difficulties encountered when studying mutant receptors. It appears that ligand binding is more dependent on an overall normal molecular conformation than on isolated or specific residues from the studies conducted so far.



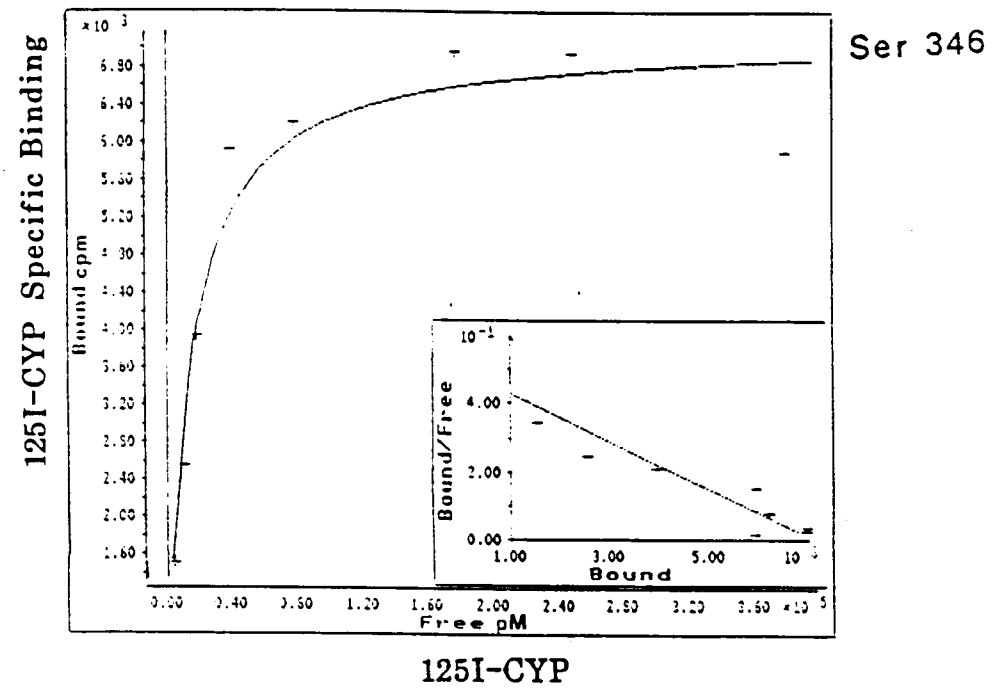


Figure 42. Saturation curves and Scatchard plots of [^{125}I]-CYP binding to Ser 346 (Ser-ala) and Ser 262 (Ser-ala) mutants.

Ser 346 Bmax: 424 fmol/mg Kd 10.5pM

Ser 262 Bmax: 184 fmol/mg Kd 10.3pM

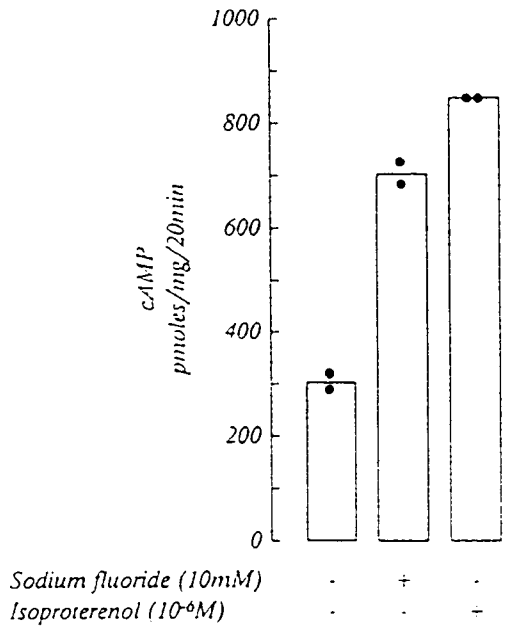


Figure 43. 10^{-6}M isoproterenol and 10mM NaF stimulation of cAMP production in B82 cells transfected with the Serine 346 (Ser-ala) mutant human beta-2 receptor gene. Bar values represent the mean of duplicate assays (•).

A clear understanding of the molecular nature of the ligand facilitates the appropriate choice of residues within the receptor for mutant constriction. Main and Tucker (1985) have emphasised the importance of the catechol ring, which might be expected to form both hydrogen bonding and hydrophobic interactions with amino acid side chains within the ligand binding pocket of the receptor.

Other residues have also been found to have important functions in facilitating normal folding of the receptor protein necessary for ligand binding, and conserved Phe residues at position 289 and 290 in helix 6 and Tyr 326 in helix 7 appear to be critical for agonist binding to the receptor. Taken together, Strader et al. (1989) suggested that residues within helices 5-7 were involved in the attachment of the catechol ring of the ligand to the beta adrenergic receptor. The model developed as a result of mutagenesis and presented by Strader is highly consistent with the results which I have obtained with the mutants.

Site directed mutagenesis of the cytoplasmic domains of the human beta-2 adrenergic receptor by O'Dowd et al. (1988) confirmed the studies of Dixon et al. (1987) suggesting that the third intracellular loop was also important in coupling to G proteins. The total picture, however will remain incomplete, until all possible mutants have been tested. It is also possible that the "negative" effect of certain deletion mutations might be due to an allosteric rather than a direct effect on the actual G protein coupling domain.

The structural basis of receptor function has been elegantly studied by Kobilka et al. (1988) using chimeric $\alpha_2\beta_2$ receptors. α_2 and β_2 adrenergic receptors are both activated by epinephrine but have opposite effects (inhibitory and stimulatory) on the adenylate cyclase system. In extensive studies of constructed chimeric $\alpha_2\beta_2$ adrenergic genes it was shown that the specificity for coupling to the stimulatory guanine nucleotide regulatory protein lies within the region extending from the 5th hydrophobic domain to the carboxyterminus of the sixth hydrophobic domain and that major determinants of α_2 and β_2 adrenergic receptor agonist and antagonist ligand binding are contained within the seventh membrane spanning domain.

By comparison with my own data and the mutant studies of Strader (1987) on the Asp 79 residue, the findings of Kolbilka et al. (1988) with chimeric $\alpha_2\beta_1$ receptors designated CR1, CR2, and CR3, in which the first and second, first second and third, and first second third and fourth hydrophobic domains were replaced by α_2 receptor sequences, still bound [^{125}I]-CYP. This result may not be too surprising, since point mutation of Asp 79 had no effect on antagonist binding. The effect however of these chimeras on agonist binding was consistent with our own study and led Kobilka to conclude that most of the hydrophobic domains influence agonist ligand binding specificity, while antagonist binding specificity is influenced primarily by the seventh hydrophobic domain, or by a combination of the 6th or the 7th. This was supported when he proceeded further and constructed "split receptors". Amazingly, co-transfection of split receptors SR 1-5 (containing only

the first five hydrophobic domains) and SR 6-7 (containing only the 6th and 7th hydrophobic domain) resulted in a functional "receptor" which bound agonists and antagonists and could activate adenylate cyclase. While chimeric receptors do provide insight into important residues for binding, there is always a concern that in constructing these receptors, molecular incompatibilities introduced might be expected to destabilise the molecule significantly making it less efficient, or even non-functional.

Dohlman (1988) has demonstrated that the beta adrenergic receptor photo affinity antagonist, p-(Bromo acetamido) benzyl-1-[^{125}I] iodocarazolol covalently binds to a peptide in the second hydrophobic domain and suggests that this domain may form or lie adjacent to the ligand binding pocket. If this domain participates directly in ligand binding, or is very closely adjacent to the ligand binding site, it is conceivable that molecules with an affinity for this domain could sterically impede ligand binding. This domain is thus a candidate site for the *in vitro* production of antibodies as a possible model to investigate the antibody mediated ligand inhibition of [^{125}I]-CYP binding discussed in Chapter 3. I have chosen this sequence as the immunogen for studies of antibody production against the beta-2 receptor discussed in Chapter 5.

The importance of phosphorylation in the regulation of the beta-2 receptors function has been studied in detail by Benovic et al. (1988) and also by Bouvier et al. (1988) who clearly showed that removal of phosphorylation sites by truncation of its serine and threonine-rich

phosphate acceptor segment delays the onset of desensitization, and that by selective replacement of 11 potential phosphate acceptor serines and threonines by glycine or alanine delays desensitisation even further.

Recently the relative importance of different receptor residues on beta-1 and beta-2 receptor ligand binding has become more clear. In studies of chimeric beta-1 and beta-2 adrenergic receptors, Frielle et al. (1988) have presented data that transmembrane region IV is largely responsible for determining binding to beta-1 vs beta-2 agonists but that regions VI and VII are important for beta-1 and beta-2 selective antagonists.

It is also clear that physical characteristics of the receptor insertion in the bilipid membrane may have significant functional importance. O'Dowd et al. (1989) have investigated the effects of mutation of Cys 341 to glycine. This resulted in a non-palmitoylated form of the receptor, which exhibited a drastically reduced ability to mediate isoproterenol stimulation of adenylate cyclase and a markedly reduced ability to form a high affinity state for agonists, suggesting that anchorage of the receptor in the membrane may also be important for normal agonist affects.

4.4 Summary and Conclusions

In this chapter, I have presented the studies which I have conducted, using molecular biology, to gain further insight into mechanisms

whereby receptor expression may be regulated (e.g. by steroids) and to gain insight into the residues of the receptor which are important for normal agonist and antagonist binding.

I was able to isolate and identify the human beta-2 receptor mRNA species and found an increase in hybridizable RNA upon induction of W1-38 cells with dexamethazone. Studies of beta-2 receptor mRNA induction in the developing lung proved difficult to perform. I constructed a 326 bp cDNA probe and several riboprobes of the beta-2 receptor gene. These probes were used to identify beta-receptor RNA species in other tissues (heart and adipose tissue) and were used in my subsequent genetic studies on the polymorphism of the receptor in asthmatic subjects (in Chapter 7).

The mutant beta-2 receptors which I studied were historically among the first mutants of the human beta-2 receptor to be constructed and they provided important information which added credibility to and strengthened proposed models of ligand binding. Studies of the human Aspartate 79 mutant were published in the Journal of Biological Chemistry (Chung et al. 1988) and Fraser (1989) has published further studies on the cysteine mutants. I have partially characterised the glycosylation (Asp 6) and serine mutants (Ser 346 and Ser 262) but further more detailed biochemical studies are required.

Studies with mutant receptors provide models for attributing functional importance to particular domains. All published mutants, however, remain informative theoretical models of beta-2 receptor

abnormalities, since spontaneous mutant beta-2 receptors have not yet been described in human disease.

The cumulative literature on point mutations of human mutant beta-2 receptors indicate that while a number of mutations could theoretically occur spontaneously *in vivo* without causing significant functional effects, there are certain residues which are critically important. Mutations at these critical residues are probably lethal. Where a mutation leads to a partial reduction in agonist binding and function (e.g. Asp 79), such a mutation, if occurring spontaneously, could theoretically underlie a disease process in which beta-2 adrenergic receptor function is impaired or more susceptible to down regulation. It would therefore be important to clone and sequence the human beta-2 receptor gene in diseases where beta-2 receptor expression or function is reported to be abnormal to identify spontaneous or inherited mutated beta-2 receptor genes in humans.

CHAPTER 5

PRODUCTION AND PARTIAL CHARACTERIZATION OF A MONOCLONAL ANTIBODY AND POLYCLONAL ANTIBODIES RAISED TO A PEPTIDE FROM A LIGAND BINDING DOMAIN OF THE HUMAN BETA-2 RECEPTOR

5.1 Introduction

Displacement of [^{125}I]-CYP binding to beta-2 adrenergic receptors by DEAE purified human immunoglobulin fractions and other human serum factors implies either competitive or non-competitive interactions of these factors with the beta-2 adrenergic receptor, or its ligand binding domain. Alternatively, high affinity binding to [^{125}I]-CYP by the serum factors may also reduce the concentration of free ligand available for receptor interaction. Any hypotheses suggesting mechanisms for the interaction of serum factors with the human beta-2 receptor itself require a working model of the molecular arrangement of the amino acid residues important for ligand binding. Genetic or acquired differences in the expression of key amino acids involved with ligand binding could theoretically enhance or reduce the effects of serum blocking factors. The experiments described in this chapter were conducted to test and expand the conceptual models of the ligand binding domain, derived in part from the mutagenesis studies in 4.3, by producing a monoclonal antibody to a 20 amino acid peptide derived from a sequence of an important ligand binding domain.

Evidence from the initial cloning and sequencing analysis of the human beta-2 receptor genes (Kobilka 1987) suggested that the human beta receptor protein is expressed as a seven transmembrane molecule analogous to rhodopsin, with seven hydrophobic regions residing within the bilipid layer (intramembranous region), 4 hydrophilic loops extending into the cytoplasm and 4 extracellularly (Fig. 35).

Binding experiments which I performed at the NIH on nine mutant beta-2 receptors described in 4.3 which were expressed in B82 cells were consistent with the suggestion that a pocket, within the hydrophobic domains in the transmembranous regions of the receptor, is important for ligand binding.

Based on the assignment of ligand binding to predominantly hydrophobic domains of the receptor, I considered production of a monoclonal antibody to the ligand binding domain an important extension of the studies in Chapter 3. In order to test whether antibodies could, in fact, be raised successfully to residues of the hydrophobic domains in the beta-2 receptor (their immunogenicity has not previously been investigated) and to probe the function of such an antibody, I have on my return to Cape Town produced both monoclonal and polyclonal antibodies to a synthetic 20 amino acid peptide derived from the protein sequence of the second transmembrane domain.

Previously, when it was proposed that ligand binding of catecholamines was to an "extracellular" domain of the receptor, it was conceivable that antibodies could interact directly with such a domain.

It is now appropriate that inhibition of ligand binding by DEAE purified immunoglobulin G fractions must be re-interpreted in the light of current models of the molecular nature of the ligand binding domain suggested by gene cloning. Since the ligand binding domain is, in fact, formed within a pocket within the seven hydrophobic transmembrane regions, direct access to the theoretical binding pocket of the beta receptor (Mr of 67 000) would seem limited. A theoretical steric inhibition could be exerted if an antigen binding domain of the immunoglobulin molecule was directed specifically at hydrophilic loops which reside on extracellular aspects of the beta receptor expressed in the cell membrane. Since deletion of the extracellular loops did not influence ligand binding in deletion mutants (Dixon et al. 1987), it is unlikely that antibodies raised to the same extracellular loops would influence ligand binding. Antibodies could induce conformational changes in the receptor via weak ionic associations, hydrophobic interactions or charge effects and they could theoretically influence ligand binding by these mechanisms.

Preliminary experiments by Dr. Claire Fraser (NIH) who raised several rabbit antibodies to extracellular domains of the beta-2 receptor, based on the model of Kobilka et al. (1987), while I was working in Venter's group in the laboratory of Receptor Biochemistry and Molecular Biology at the N.I.H. showed that rabbit polyclonal antibodies to the extracellular loops did not inhibit ligand binding to any significant degree (less than 30% inhibition). Since these experiments indicated that antibodies directed to the extracellular loops of the beta-2 receptor were probably not the cause of the degree of inhibition found with the human sera, I

decided to study antibodies directed at hydrophobic regions. The effects of antibodies directed at hydrophobic domains of the beta-2 receptor have not been previously investigated.

Based on published information about the ligand binding domains, I selected the second transmembrane domain of the beta-2 receptor as an initial target for monoclonal antibody production because

(i) the deletion of amino acid residues 70-96 removed normal [^{125}I]-CYP ligand binding in the mutant constructs of Dixon (1987).

(ii) Residue Asp-79 was shown to be important in agonist binding by Strader (1987).

(iii) Residue Aspartate 79 was also thought to be important in G protein binding in the mutant studies work I conducted in Chapter 4.

(iv) Amino acid residues 83-96 in helix 11 were shown by Dohlman (1988) to contain the site of p-(Bromo acetamido) benzyl-L-[^{125}I] Iodocarazolol binding.

It was envisaged that successful production of a monoclonal antibody directed to this ligand binding domain could be used as a control antibody and a model for studies exploring the interactions of human immunoglobulin with ligand binding (if in fact antibody was able to enter the ligand binding domain). Such an antibody could also theoretically be used:-

(i) to study the effects of anti-beta-2 receptor antibody on receptor function and expression in cultured cells;

(ii) to identify beta-2 receptors in tissue sections using immunofluorescence or colloidal gold;

(iii) to identify expressed mutant beta-2 receptors which did not bind beta receptor ligands; and

(iv) as an affinity probe to study post-translational processing and membrane insertion of the beta-2 receptor in cultured cells.

5.2 The 20 amino acid second transmembrane beta-2 adrenergic receptor peptide BR-20-II.

The peptide chosen for monoclonal antibody production comprised 20 amino acids derived from the 2nd transmembrane domain of the human beta-2 receptor in the following sequence:

Asp^{79} Leu Val Met Gly Leu Ala Val Val Pro Phe Gly Ala Ala His Ile Leu Met Lys Met^{99}

Residues extending extracellularly to Met^{99} were included to facilitate possible binding of the antibody to the receptor in the intact molecule.

The location of this peptide in the transmembrane model of Kobilka et al. (1987) is shown in Fig. 44.

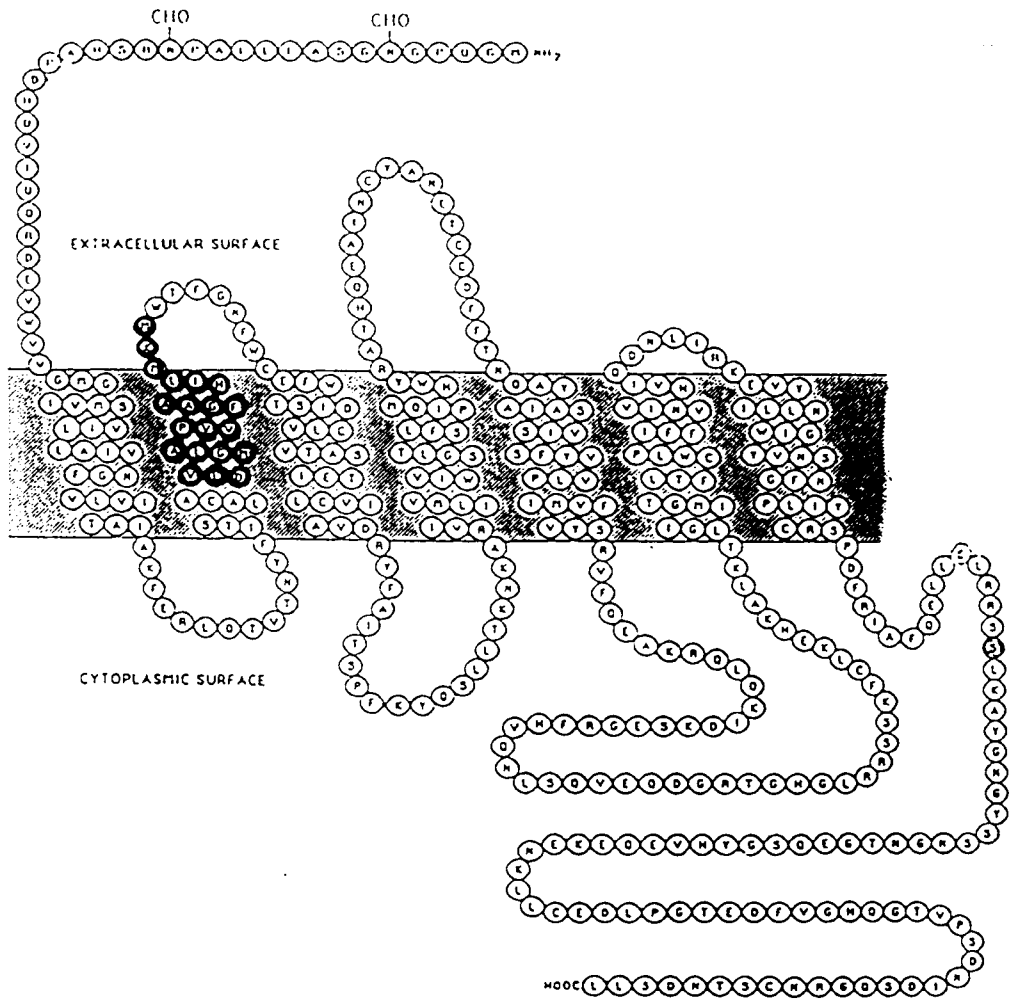


Figure 44. Location of the 20 amino acid hydrophobic peptide sequence in the second transmembrane domain of the human beta-2 receptor (using a model adapted from O'Dowd et al (1989).

For the purposes of immunization, at my request, three peptides were synthesized, using the Fmoc polyamide synthesis method (Kemp 1979), in the Department of Biochemistry at the University of Pretoria by Marina Rautenbach.

(1) Peptide I

Asp^{79} - Met^{99}-Cys(ACM)-Gly (BR-20-I)

(2) Peptide II

N-Palmitoyl Asp^{79} - Met^{99}-Cys(ACM) Gly (BR-20-II)

(3) Peptide III

N-Cys Asp^{79} - Met^{99}-Cys(ACM)-Gly (BR-20-III)

All three peptides were highly insoluble in aqueous solvents because of their hydrophobic nature and their synthesis was chemically difficult (Rautenbach 1989).

Peptide II (BR-20-II) was selected for the initial immunization, since by coupling a palmitoyl residue to the N-terminus of the peptide, direct immunization was possible (no deblocking of ACM-Cys or adjuvant was required). Since the enhanced immunogenicity of lipid-conjugated protein was discovered and reported by Stark (1980), it has been shown by Hopp (1984) that a HBsAg peptide-palmitic acid conjugate produced a 27.4 fold titre increase (compared with a 4.6 fold titre increase following immunization with a KLH derivative) and that there is no antibody response to the fatty acid portion of the dipalmitoyl conjugate. It is believed that palmitoyl-conjugated peptides form a micelle or stable aggregate corresponding to between 50 and 1000 copies of the conjugate per particle and that the dipalmitoyl peptide effectively acts as its own carrier. Amino acid analysis of this peptide supplied by Prof. L. Visser (University of Pretoria) is shown in Appendix H.

5.3 Immunization

Three six week old BALB/c mice were immunised intraperitoneally with 100μg of an aqueous suspension of the palmitoyl peptide (BR-20-II) and boosted again with 50 μg of peptide after four weeks and again at six weeks. No adjuvants were used. Three days after the second boost the mice were test bled from the retro-orbital plexus. The serum was separated, aliquotted and frozen at -20°C.

5.4 Screening Assay

I developed an ELISA test to screen sera, harvest fluids and ascites for anti-beta-2 receptor peptide antibody production. In order to optimise the conditions for the ELISA I conducted chequer-board experiments to standardise antigen concentrations, solvents, microtitre plates and antibody concentrations.

In view of the insolubility of the BR-20-II peptide in aqueous solutions, peptides were dissolved in 100% methanol or dimethyl sulphoxide (DMSO). Ninety-six well Titertek (plastic) Seravac plates were compared with 96-well Microelisa$^{(R)}$ (Greiner) polystyrene plates. Plates were coated overnight (14-16 hrs) and air-dried with BR-20-II peptide concentrations varying from 0.5μg-2μg/well.

For detailed description of the ELISA reagents see Appendix I. Briefly, after an overnight coating with the peptide in methanol (50μl), the plates were washed five times with phosphate buffered saline (PBS), blocked for

one hour with 200μl 1% fetal calf serum in PBS and then incubated for one hour at room temperature with 50μl of dilutions of the mouse serum (or hybridoma fluid). After 10 further washes with Tris saline Tween (TST) the plates were incubated with a 100μl of a 1/500 dilution of goat anti-mouse conjugate in ELISA diluent, washed five times with TST after which 100 μl of ABTS substrate was added. Absorbance at 414 nM was read after 30 min.

I found that Titertek plates gave higher readings and less background than the MicroElisa(R) plates. DMSO was an unsuitable solvent because it not only formed an opaque reaction on the Titertek plates, but doubled the background on the polystyrene plates. A peptide concentration of 0.5μg/well gave absorbance readings between 0.5 - 1.0 with a 1:100 dilution of the mouse serum and was used for all subsequent peptide ELISAs.

Using the BR-20-II peptide antibody ELISA, all three mice were test bled after the second intraperitoneal boost for antibody production to the peptide. At 1/100 dilution of mouse serum, absorbance 414nm readings between 0.310 and 0.618 were obtained for all three mice (background readings 0.132-0.242). The BALB/c mouse whose serum gave the highest readings in the ELISA was selected for the fusion.

5.5 Fusion and Cloning

Chemical reagents, cells and media used for the production of the monoclonal antibody are detailed in Appendix J.

Three days prior to fusion, the BALB/c mouse was boosted with 100μg peptide BR-20-II intraperitoneally. SP2 myeloma cells were thawed from a frozen stock and cultured in 10% FCS in RPMI with antibiotics. Cells were plated out in 35mm petri dishes at 10^5 cells/ml and passaged in 100mm petri dishes prior to the fusion. On the day of fusion supernatant medium was removed from a 100mm dish (approximately 10^7 cells in mid exponential phase) and 10ml of 1x Versene solution was added. After 10 min incubation at 37°C the cells were dislodged by pipetting and transferred to a 50 ml centrifuge tube, centrifuged at 1500 rpm for 5 min (using a MSE minor bench top centrifuge) and resuspended in 2ml RPMI (without antibiotics) and viability checked under phase contrast microscopy, using Trypan blue.

The immunised BALB/c mouse was killed using ether, the abdomen was sterilised with 70% alcohol. The spleen was removed and placed in RPMI, loose connective tissue was dissected off and the spleen was transferred to a clean dish, and the spleen was squeezed through a sterile stainless steel sieve into RPMI with the plunger of a 2 ml syringe. Clumps were broken by pipetting the suspension a few times and the larger pieces were allowed to settle for a few minutes before the supernatant was transferred to clean tubes. Cells were washed once with RPMI and diluted in white cell counting fluid.

For the fusion, 6 x 10^7 spleen cells were mixed with 1 x 10^6 myeloma cells in plain RPMI and suspended in 30 ml RPMI in a 50 ml centrifuge tube and centrifuged at 1000 rpm (MSE minor) for 5 minutes. The supernatant was poured off and after loosening the pellet, 1.5 ml PEG was

added dropwise over one minute with gentle mixing, stirred gently for a further minute after which 1 ml of warm 37°C RPMI was added slowly over one minute with gentle mixing, a further 1 ml RPMI over the next minute, followed by 8 ml over three minutes and then topped up to 45 ml, centrifuged again at 1000 rpm for 3 min and the supernatant removed. The pellet was suspended in 50 ml HAT-HUCS and distributed in 0.5ml aliquots into 24 well plates after which another 0.5ml HAT-HUCS was added. Supernatants were tested for antibody production after 14 days, when they started to turn yellow, using the peptide ELISA.

Three positive clones were obtained and the strongest positive, well AA6, was cloned by limiting dilution in HT-HUCS in 96-well flat-bottom microtitre trays. Most of the clones were positive in the peptide ELISA and I selected clone B4 for expansion and further cloning by limiting dilution. All of these clones were positive and clones B4A8 and B4E4 were chosen for ascites production and characterization. A selection of positive clones were frozen down and stored in liquid N_2. Clone B4A8-PWL was expanded in 100mm dishes in 10% FCS and 5 x 10^6 cells in 0.5ml PBS were injected into BALB/c mice which had been pristane (tetramethylpartidecane) primed one week previously and ascites tapped 10 days later. Harvest fluids from 100mm dishes were stored in aliquots at -20°C.

5.6 Characterization of B4A8-PWL

5.6.1 Titre in Peptide ELISA.

Using the peptide-immunised BALB/c mouse serum and neat harvest fluid from seven clones as positive controls and control mouse serum and an irrelevant monoclonal anti-Erythrina trypsin inhibitor (ETI) as negative controls, ascites from clone B4A8-PWL was tested in the peptide ELISA in dilutions 1:100 to 1:48 000 (Fig. 45). Strongly positive titres were obtained down to a titre of 1:24 000 (Absorbance 414nm readings greater than 0.33 were regarded as positive). The dilution of the antiserum providing a half maximal signal for the immobilised peptide was approximately 1:4000. (Mean absorbance with irrelevant monoclonal 1:200 dil. = 0.295). Positive clones from B4A2 were also tested in the peptide ELISA and titres of 1:800 were obtained.

5.6.2 Inhibition of B4A8-PWL binding in ELISA by pre-incubation with the peptide.

In order to demonstrate that the monoclonal B4A8-PWL could bind to free peptide and not only to the peptide coupled to the microtitre dish, serial dilutions of antibody were pre-incubated with increasing concentrations of beta receptor BR-20-II peptide prior to the ELISA. A 1:4000 dilution of B4A8-PWL ascites gave maximum inhibition when pre-incubated with increasing concentrations of BR-20-II (Fig.46). Inhibition of more than 50% was not obtained at the peptide

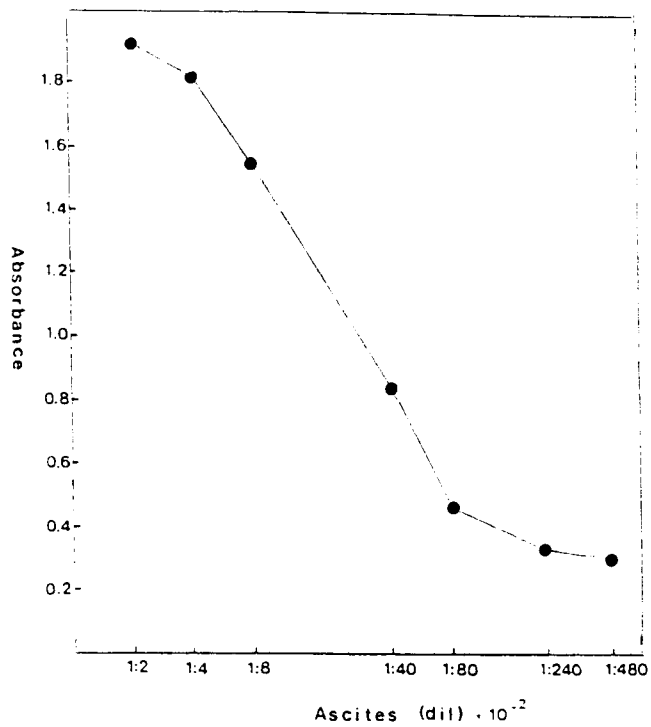


Figure 45. Binding of monoclonal B4A8-PWL to BR-20-II peptide attached to microtitre plate. (Mean absorbance for blank with no antigen = 0.24; mean absorbance for 1:100 dilution of immunised mouse serum = 0.51; mean absorbance for neat B4A8 harvest fluid = 0.92).

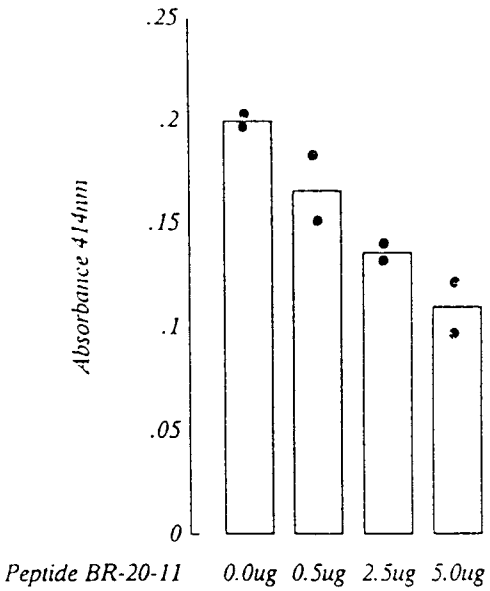


Figure 46. Inhibition of binding of monoclonal B4A8-PWL to BR-20-II in peptide ELISA by pre-incubation overnight with 0.5μg, 2.5μg and 5μg of BR-20-II peptide at a 1:4000 dilution of B4A8-PWL. Bars represent mean values of duplicate ELISA assays (•).

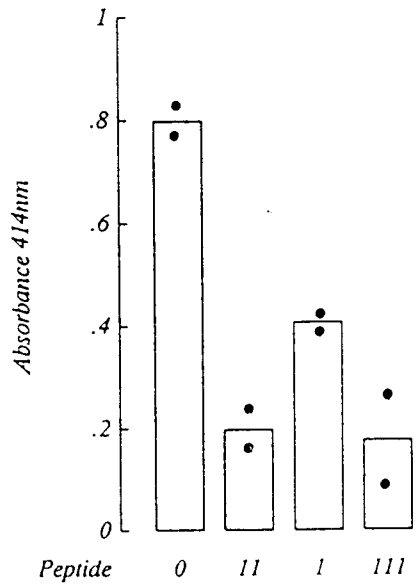


Figure 47. Inhibition of binding of monoclonal B4A8-PWL to BR-20-II in peptide ELISA by pre-incubation with 5μg of BR-20-I, BR-20-II and BR-20=III at a 1:4000 dilution of B4A8-PWL. Bars represent mean values of duplicate ELISA assays (•).

concentrations tested. These experiments showed that the monoclonal recognised and bound to the free peptide which was present as an aggregate or micelle. In a separate experiment inhibition of antibody binding with peptides (5μg) BR-20-I and BR-20-III was also demonstrated (Fig.47), supporting the conclusion that the monoclonal B4A8-PWL recognises an epitope common to all three of the synthesized beta receptor peptides and is thus not directed against the palmitoyl residue of BR-20-II.

5.6.3 Isotyping of B4A8-PWL

B4A8-harvest fluid was concentrated 16x using a Centricon (Amicon). Isotyping was performed using rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA antibody (Miles). Briefly, 2μl of isotyping sera diluted 1/20 was spotted on to nitrocellulose paper which had been previously wetted and allowed to dry. After the nitrocellulose paper was immersed in 5% fetal calf serum in TBS (with 0.5% Tween 20) for 30 minutes, 5μl of monoclonal antibody harvest fluid was applied to each spot and allowed to dry for 3-5 min, washed twice in TBS for a few minutes, and peroxidase conjugated goat anti-mouse antibody applied for 5 minutes, washed 2x with TBS and immersed in 4 chloronaphthol substrate for 15 minutes.

Positive spots were obtained with rabbit anti-mouse IgM and an Ouchterlony test confirmed that the isotype of the anti-peptide monoclonal B4A8-PWL was in fact IgM.

5.6.4 Recognition of a 49 kDa expressed protein by B4A8-PWL.

In order to determine whether the anti-peptide monoclonal B4A8-PWL recognised a biological molecule, Western blots of membranes of mouse fibroblasts (B82 L cells) which I had transfected with the human beta-2 receptor gene (6.2) expressing high levels of beta-2 receptors (4000 fmol/mg protein Wt-8 Cape Town clone) were compared with membranes from non-transfected control B82 L cells (not expressing detectable beta-2 receptors) according to the method of Towbin et al. (1979).

Briefly, 100μg of membranes were prepared according to the detailed method described in Appendix F. Membranes containing 26ng beta-2 receptors (measured by saturation curves and Scatchard plots) were separated electrophoretically on PAGE (9%) and blotted on to Immobilon™ Polyvinylidene difluoride (PVDF) microporous membranes which had been pre-wet with methanol.

After blocking with 5% FCS and 0.3% Tween 20 for 2 hours, the Western blots were incubated with a 1/1000 dilution of the monoclonal antibody B4A8-PWL for 1 hour, washed 5 times with TBS in 0.05% Tween 20 and then incubated with sheep anti-mouse IgG 1/200 diluted in TBS for 30 min. After washing 3 times it was incubated again with PAP (peroxidase anti-peroxidase) 1/20 in TBS for 30 minutes, washed 5x with TBS and incubated with DAB (Diaminobenzamidine 1/50, H_2O_2 1/1000 and cobalt 0.3%) for a few minutes and then washed with H_2O and allowed to dry. The use of this sandwich technique (Lansdorp et al. 1984) using the sheep anti-mouse

antibody as a linker was found necessary to eliminate unacceptable levels of non-specific binding which I originally encountered when a peroxidase-conjugated goat anti-mouse antibody was used. In further experiments, 5% fetal calf serum was found to be more effective than 10% horse serum or 1% gelatin as a blocking agent using both PAP and APAAP (Hohmann et al. 1988) systems, with the sheep anti-mouse linker.

Results of the Western blots of reduced membranes (100μg/lane) showed that the B4A8-PWL monoclonal recognised 49 kDa protein present in both transfected and non-transfected mouse B82 L cells (Fig. 48). The 49 kDa protein was not present on Western blots of lysates of human red blood cells, but recognised as a single band by the monoclonal antibody B4A8-PWL in Western blots of PAGE-separated lysates of the UCT-BR-1 breast tumour cell line (Wilson 1982), Jurkat T cells, Bowes melanoma cells (Harris 1986) and in human B cells. In human B cells an additional lower band, approximately 40 kDa, was also detected.

The intensity of the band did not differ when Western blots of B82 L cells expressing different numbers of beta-2 receptors (400 fmol/mg - 4000 fmol/mg) were compared suggesting that the 49 kDa protein was not in fact related to the human beta-2 receptor. Although there was no visible recognition of the expressed human beta-2 receptor protein by the monoclonal B4A8-PWL, this does not completely rule out possible binding to the receptor since only 26ng of human beta-2 receptor was present in each lane. Wang (1989) used 200ng of purified beta receptor from mouse lymphoma in their immunoblots incubated with antipeptide sera and it is

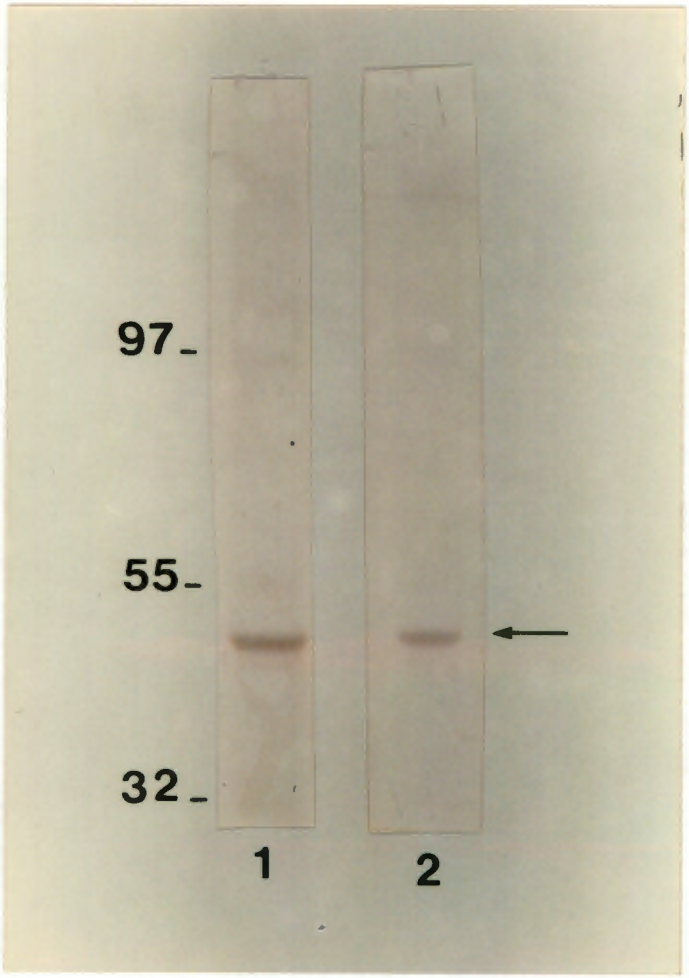


Figure 48. Western blot of membranes from non-transfected mouse cells (lanel) and membranes from clone Wt-7 Cape Town (lane 2) probed with monoclonal B4A8-PWL (1:500 dil.) showing recognition of a 49 kDa band in both cell membrane preparations. Molecular weights are shown on the left (kDa).

possible that insufficient receptor may be present in my Western blots for detection using this system.

5.6.5 Immunofluorescence Studies

In order to investigate more precisely the topography of the 49 kDa protein recognised by B4A8-PWL in Western blots of membrane preparations of the B82 cells, and to investigate whether beta-2 receptors could be detected with B4A8-PWL, indirect immunofluorescence studies on intact cells were performed. The cell lines, and culture conditions are described in detail in Appendix E.

B82 cells expressing high levels of beta-2 receptors (Clone Wt-7 Cape Town 4000 fmol/mg) and control non-transfected B82 cells were cultured on glass slides in the presence of dexamethazone (10^{-6}M) and immunofluorescence of fixed and unfixed cells with the B4A8-PWL monoclonal antibody was examined under UV light. The protocol used for the immunofluorescence studies is described in Appendix K.

Positive fluorescence was only seen when fixed (permeabilised) cells were used, but both transfected (Wt-7 Cape Town) and non-transfected cells fluoresced with B4A8-PWL. The pattern of fluorescence was that of a fine speckling (Fig. 49(a)) distributed towards the periphery of the cells and quite unlike the homogenous fluorescence seen when a control monoclonal, anti-ETI was used on the Wt-7 Cape Town cells using the same conditions (Fig. 49(b)). Capping was seen on some cells.

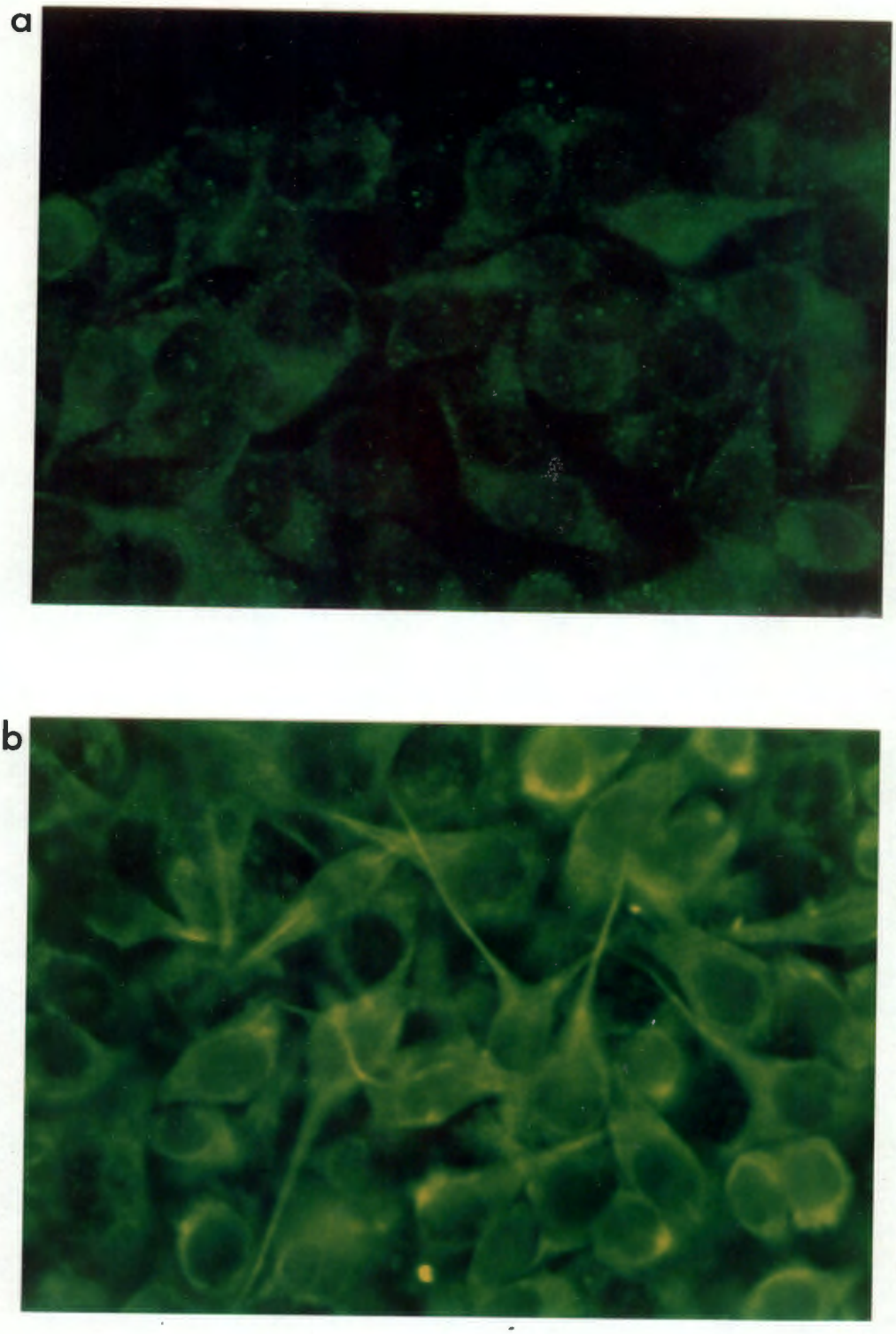


Figure 49. Speckled immunofluorescence seen with fixed transfected cells (clone Wt-7 Cape Town) with monoclonal B4A8-PWL (49a) compared with the homogeneous immunofluorescence observed when a control monoclonal (anti ETI) was used on the same cells (49b).

These studies and the studies of membranes on the Western blots suggested that the 49 kDa protein recognised by B4A8-PWL is expressed in discrete locations in an intramembranous location. If there was any binding to beta-2 receptors, this was completely obscured by fluorescence of the more abundant 49 kDa protein which was present in both transfected and non-transfected cells.

5.6.6 Immunoabsorption.

The relationship between the anti-peptide II antibody B4A8-PWL and the 49 kDa protein was further investigated in a series of immunoabsorption experiments.

Prior to conducting the peptide ELISA (Appendix I) B4A8-PWL ascites (1/200 dil.) were pre-incubated with 25, 50, 100, 200 and 800μg of B82 (high and low beta-2 receptor expressing) cell membranes overnight at 4°C, using a Braun shaker. The membranes were then pelleted using a Beckman air-driven ultracentrifuge for 5 minutes at 30 psi and the supernatants were tested in the peptide ELISA. An anti-ETI monoclonal antibody was used as a negative control. Both transfected and non-transfected cells absorbed out anti-peptide activity. A dose response of the absorption is given in Fig. 50. No absorption was found with the irrelevant monoclonal antibody anti-ETI, supporting the conclusion that the peptide and the 49 kDa protein share a common epitope. The high beta-2 receptor expressing cell membranes did not absorb out more anti-peptide activity than the low expressing cells, consistent with my original findings with the Western blots and provided further evidence that the 49 kDa protein

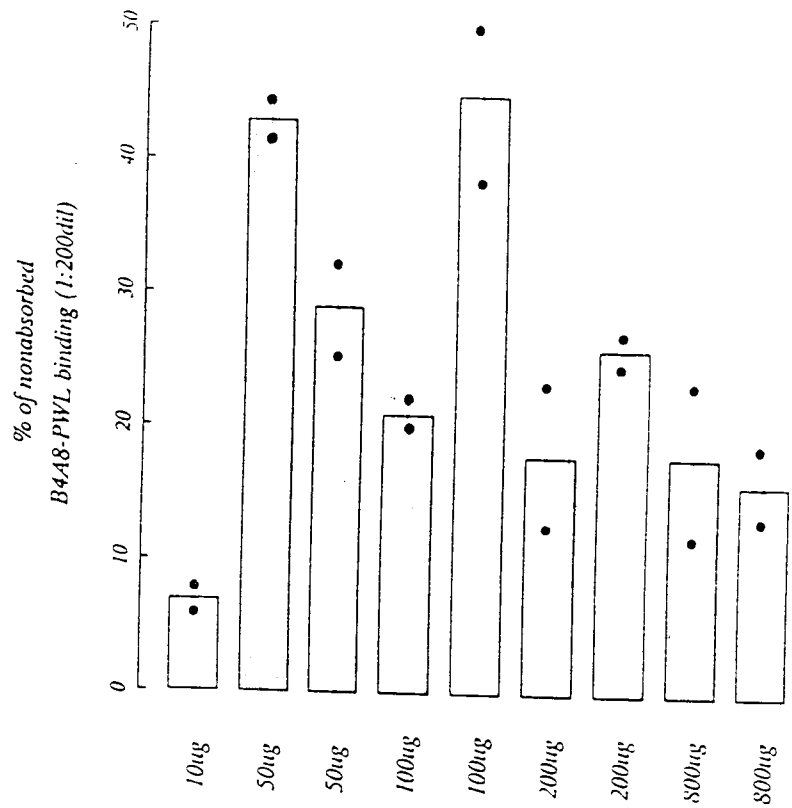


Figure 50. Binding of B4A8-PWL which had been pre-absorbed to 10μg, 50μg, 100μg, 200μg, 800μg of membranes from untransfected B82 cells (▩), or Wt-7 Cape Town cells (□), compared with binding of non-absorbed B5A8-PWL (1:200 dil). (Bar values represent the mean of duplicate experiments (.)).

was not a processed or expressed human beta-2 receptor, and that the monoclonal B4A8-PWL did not recognise the beta-2 receptor.

In order to confirm what was previously assumed, i.e. that the non-transfected B82 cells could not be expressing a processed or non-functional human beta-2 receptor, I prepared genomic DNA (based on methods I have described in 7.4) from confluent cultures of transfected (Wt-7 Cape Town) and non-transfected B82 cells. 12 μg of DNA from each culture was digested with Pst-I for 2 hours and then Southern blotted on to Hybond N prior to probing with a nick translated [^{32}P]-labelled 2.6kb genomic probe (see Chapter 4.2.3) of the human beta-2 receptor. The absence of restriction fragments on the autoradiograph of the non-transfected cells (shown in Fig. 51) demonstrates absence of the beta receptor gene in the non transfected B82 cells. No explanation has previously been reported for the absence of expression of beta-2 receptors in the B82 cells. These experiments provided firm evidence that the 49kDa protein was not in fact a precursor of or processed form of the human beta-2 receptor.

5.6.7 Biological activity of B4A8-PWL Monoclona Antibody.

(a) Effects on human fibroblast growth.

Biological activity of the B4A8-PWL Monoclonal antibody (Mab) was investigated using human non-transformed foreskin fibroblast cultures established from foreskins obtained from neonatal circumcision (a gift from Dr. Colleen Fearns, UCT). Using Scatchard plots of [^{125}I]-CYP

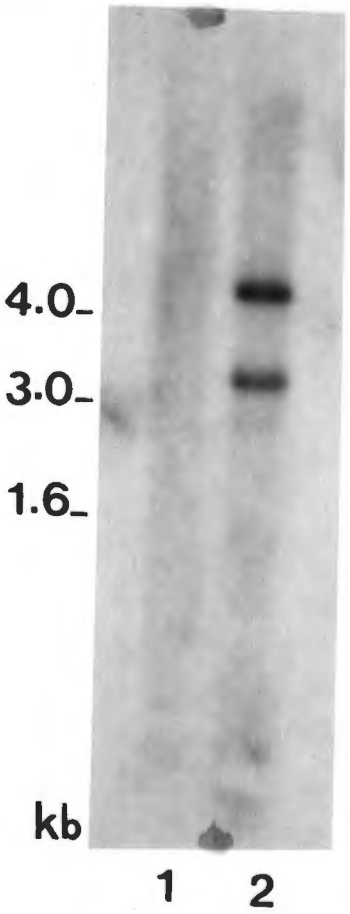


Figure 51. Autoradiograph of a Southern blot of genomic DNA prepared from confluent cultures of non-transfected B82 cells (left lane) and B82 cells transfected with human beta-2 receptor gene (clone Wt-7-Cape Town)(right lane) digested with Pst-1 and probed with a nick translated [^{32}P] labelled 2.6 kb genomic probe of the human beta-2 receptor gene (Stringency 0.1xSSC, 0.1% SDS at 65°C for 2 hours).

binding, foreskin fibroblasts expressed approximately 100fmol beta receptors/mg membrane (Kd 30 pM). Growth curves of the foreskin fibroblasts revealed a doubling time of about 48 hours.

Preliminary studies examined the effect of beta-2 blockade and beta-2 stimulation on the growth rate of the foreskin fibroblasts using 10^{-6}M L(-) propranalol and 10^{-6}M ± isoproterenol. Culture of the fibroblasts under these conditions showed a retardation of fibroblast growth at 48 hrs in the presence of 10^{-6}M ± isoproterenol and an enhancement in the presence of 10^{-6}M L(-)propranolol. When the human fibroblasts were cultured in the presence of B4A8-PWL Mab 1/400 dilution (ascites) there was a 46% reduction in cell proliferation. No inhibition of growth was observed with a 1/400 dilution of an irrelevant monoclonal (αIgE antibody) ascites (Fig. 52). These experiments showed that binding to the 49kDa protein by the monoclonal B4A8-PWL resulted in a functional effect on cell proliferation, namely inhibition, but since the 49kDa protein bore no relation to the beta-2 receptor I did not study these inhibitory effects further. I have no explanation for the apparent stimulatory activity observed in the presence of L(-)proranolol.

(b) Inhibition of the ^{125}I-CYP binding to the beta-2 receptor by B4A8-PWL.

Since I originally made the monoclonal B4A8-PWL with a view to using it as a control antibody for ligand inhibition experiments, I proceeded to to investigate possible interference of B4A8-PWL on [^{125}I]-CYP with ligand binding, even though experiments I had performed thus far suggested no direct interaction with the beta-2 receptor.

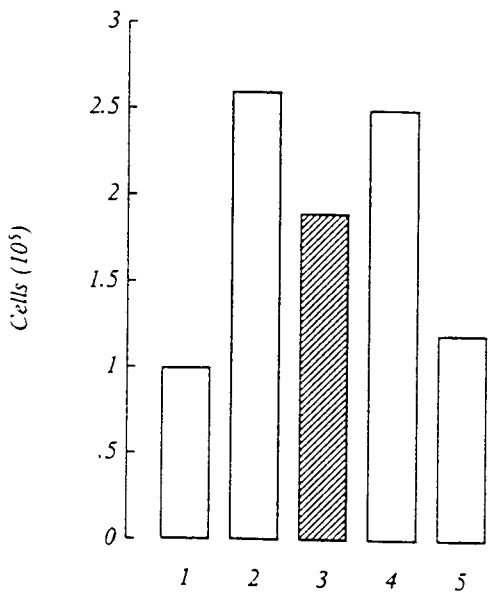


Figure 52. Growth of human foreskin fibroblasts (number of cells x 10^5) cultured for 72 hours in RP-10 alone (Column 2), RP-10 with B4A8-PWL (1:400 dil.)(Column 3), RP-10 with irrelevant anti-IgE ascites (1:200) (Column 4), and RP-10 with 10^{-6}M isoproterenol (Column 5)(Number of cells at zero time shown in Column 1). Bar values represent mean number of cells counted in duplicate plates.

Although B4A8-PWL Mab recognised a 49 kDa protein on Western blot and did not apparently recognise glycosylated (69 kDa) or unglycosylated (43 kDa) forms of the beta receptor (bearing in mind that only 26ng receptor was present per lane), I thought it would still be interesting to investigate whether there was any specific or non-specific interaction with the ligand binding site of the beta-2 receptor when the receptors were functionally intact within the membranes i.e. it was conceivable that the monoclonal could recognise conformational epitopes in the intact molecule, but not sequential determinants in the Western blot.

In the first of a series of experiments, the effect of the B4A8-PWL Mab on antagonist binding was examined using the [^{125}I]-CYP ligand binding assay (Chapter 2.2.3). B4A8-PWL Mab ascites was used at a 1/50 dilution. Initial inhibition assays suggested that incubation with the Mab did result in mild inhibition (less than 30%) of [^{125}I]-CYP binding, but that the degree of inhibition varied with the membrane preparation used. Inhibition was tested through a range of dilutions of the monoclonal antibody. The effect of receptor number in the membranes and degree of inhibition by the monoclonal was formally tested in experiments in which inhibition by B4A8-PWL (1/50 dilution) was measured using three different B82 membrane preparations, with three levels of human beta 2 receptor expression and compared with the inhibition obtained with a 1/50 dilution of an irrelevant monoclonal (αIgE Mab) in these membranes. The results of this experiment were highly reproducible and confirmed that a relatively greater inhibition occurred with membranes from B82 cells expressing lower levels of human beta-2 receptors (488 fmol/mg)(27%) than with membranes from B82 cells expressing high levels of human beta-2 receptors

(4203 fmol/mg) (9%), but surprisingly the irrelevant monoclonal αIgE Mab produced inhibition of the same order (Fig. 53). This experiment demonstrated convincingly that low degrees of beta receptor ligand inhibition (<30%) can be mediated in the ligand binding assay by dilutions of irrelevant immunoglobulins which are not directed against the ligand binding site and further confirmed that B4A8-PWL did not interact with the ligand binding domain to any significant degree. This finding (see Chapter 2.7) indicated that the ligand inhibitory effects of sera *in vitro* may be mediated by a combination of factors which may include immunoglobulins with ligand specificity or receptor specificity but can also contributed to by, irrelevant immunoglobulins and other non-immunoglobulin fractions which are also present in dilutions of the ascites fluids.

B4A8-PWL IgM was also purified from the ascites fluids using Sephadex G200 chromatography. Five fractions containing IgM were identified by dot blotting using the method described in 5.6.3. None of these fractions inhibited [^{125}I]-CYP binding in the ligand binding inhibition assay of greater then 25%, consistent with the experiments conducted with dilutions of the ascites fluids.

5.6.8 Immunoprecipitation of the 49 kDa protein by B4A8-PWL

Specificity of B4A8-PWL was investigated further in a series of immunoprecipitation experiments. It was important to investigate whether B4A8-PWL Mab could immunoprecipitate the 49 kDa protein and to see if the B4A8-PWL could simultaneously recognise and co-precipitate a

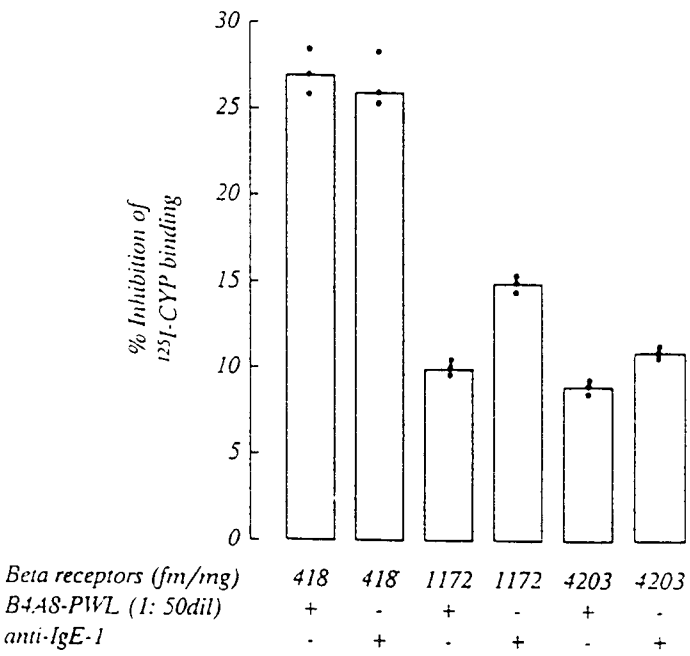


Figure 53. % inhibition of [^{125}I]-CYP binding by 1:50 dilution of B4A8-PWL, or irrelevant anti-IgE, to membranes prepared from 3 different clones of B82 cells transfected with the human beta-2 receptor gene expressing 418 fm/mg, 1112 gm/mg and 4203 fm/mg human beta-2 adrenergic receptors. Bar values represent the median value of experiments performed in triplicate (.).

conformationally intact beta-2 receptor when solubilised from its membrane constraints, when access to the receptor by the antibody was theoretically easier.

B82 cells transfected with the beta-2 receptor gene were cultured in methionine-free medium to semi-confluence prior to pulsing with 10^{-6}M dexamethazone. Six hours prior to harvesting, 25μCi of [^{35}S]-methionine (Amersham) was added to the dish. Membrane preparations of the cells were made (Appendix F) and protein concentrations determined by the Lowry method.

To solubilise the membrane proteins, 6 mg membrane protein, in 1.0 ml 20mm sodium phosphate 2mm magnesium sulphate buffer was incubated in 0.5% Triton X-100 for 15 min at 40°C. Unsolubilised protein and membrane pellets were removed by centrifugation at 100 000g in 150μl aliquots for 10 min using an airfuge and the supernatant was treated with Biobeads SM-2 (Biorad) 2g/ml for 30 min at 4°C to remove detergent. The supernatant was then removed from the Biobeads by gentle pipetting and incubated with a 1:10 dilution of B4A8-PWL (ascites) overnight at 4°C.

200μl of a solution containing cyanogen bromide-activated sepharose coupled to sheep anti-mouse antibody, freshly prepared (see Appendix L), was added and tumbled for 2 hours at room temperature. After washing 5 times with PBS using a microfuge, 20μl of sample buffer containing mercaptoethanol was added, the sample boiled for 2 minutes and after centrifuging in a microfuge for 10 min the supernatant was electrophoresed on 9% PAGE, Western blotted on to Immobilon™ and

autoradiographs were incubated using Cronex 4 X-ray film for 48 hours at -70°C.

Results of the autoradiograph shown in Fig. 54 show clear precipitation of the 49 kDa protein as well as a high molecular weight protein greater than 97 kDa. There was no evidence on the autoradiograph, of precipitation of the solubilised human beta-2 receptor, even after prolonged autoradiography (7 days).

I also attempted to immunoprecipitate solubilised [^{125}I]-CYP-labelled beta-2 receptors directly with the B4A8-PWL monoclonal antibody. Wt-7 Cape Town clone membranes were incubated with 0.5% digitonin for 30 min at 30°C and then centrifuged at 40 000g for 60 min to pellet the membranes. Solubilised receptors were measured after incubation of the supernatant with 30 pM [^{125}I]-CYP for 30 min at 30°C. Solubilised receptors were precipitated on Whatman GF/B filters after the addition of 15% PEG and γ-globulin. Although propranalol-displacable specific binding was present in the polyethylene glycol precipitates, only non-specific binding was precipitated when solubilised receptors were incubated with varying dilutions of B4A8-PWL (without PEG), followed by incubation overnight with sheep anti-mouse antibody at 4°C (Fig. 55).

The collective data acquired independently from the Western blots, the [^{125}I]-CYP inhibition assays and immunoprecipitation assays provided no evidence for recognition of the human beta-2 receptor by B4A8-PWL monoclonal antibody but specific binding to the 49 kDa protein is

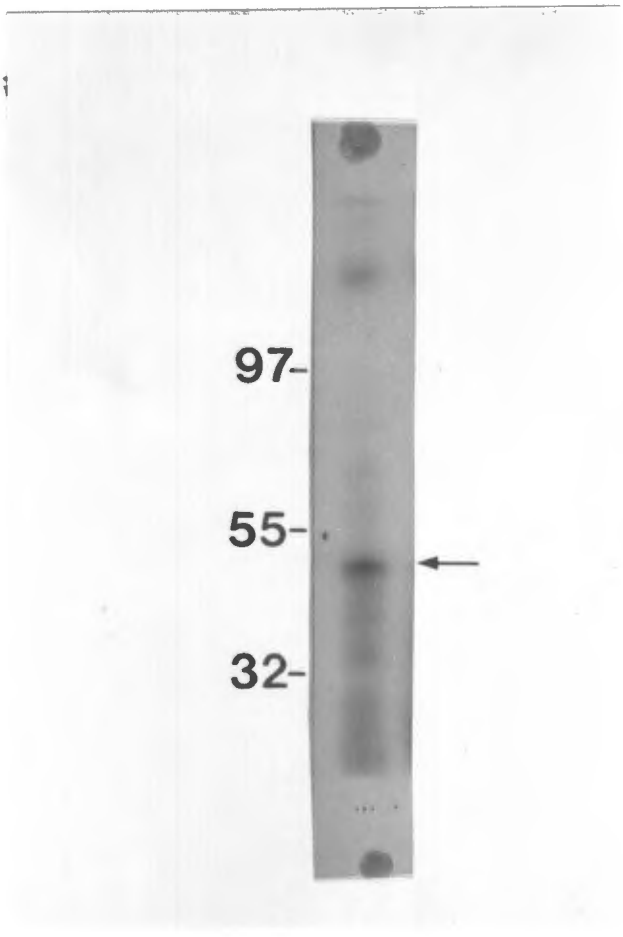


Figure 54. Autoradiograph of the immunoprecipitation of a 49 kDa band from solubilised membrane preparations of [^{35}S]-methionine pulsed Wt-7 Cape Town cells using B4A8-PWL monoclonal and sheep anti-mouse sepharose (exposure 48 hours, -70°C, Cronex 4 Xray film).

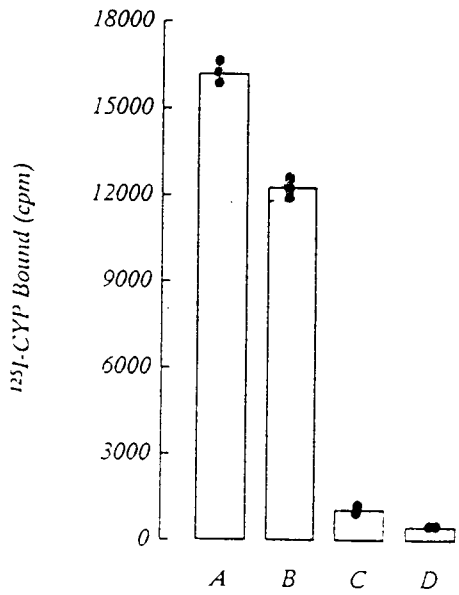


Figure 55. Precipitation of human beta-2 receptors solubilised using 0.5% digotonin in the presence of 15% polyethelene glycol and -globulin (Column B) or B4A8-PWL monoclonal antibody (Column C). Total [^{125}I]-CYP (cpm) in solubilised receptors in the absence of (-)L propranalol is shown in Column D (non-specific binding). Column A represents total number of receptors in membrane preparation prior to solubilization. Bar values represent the mean of assays performed in triplicate.

consistently obtained. Characterization of the B4A8-PWL monoclonal is summarised in Table 5.6.9.

5.6.9 Summary of the characterisation of the B4A8-PWL monoclonal antibody.

1. Recognition of BR-20-II peptide in ELISA to a titre of 1:24000 (Fig. 45)
2. IgM isotype (dot blot and Ouchterlony)
3. Pre-incubation of B4A8-PWL with BR-20-II peptide inhibits binding in ELISA (Fig. 46).
4. Strong recognition of a 49kDa protein in a Western blot of both β receptor-transfected and non-transfected L cells (Fig. 48).
5. Recognition of the 49kDa protein in UCT BR-1 cells, Jurkat cells, Bowes melanoma cells and human B cells, but not red cells.
6. Positive speckled immunofluorescence only in fixed (permeabilised) transfected and non-transfected B82 cells (Fig. 49).
7. Immunoabsorption studies confirmed that the synthetic peptide and 49kDa biological protein shared a common epitope (Fig. 50).
8. Biological activity of the 49kDa protein was suggested by incubation of the cells with a 1:200 dil of B4A8-PWL which resulted in a 46% reduction in cell proliferation (Fig. 52).
9. Low degrees of [^{125}I]-CYP inhibition (up to 30%) to human beta-2 receptors were obtained by a 1:50 dilution of B4A8-PWL and the degree of inhibition was related to the level of beta-2 receptor expression.

10. Immunoprecipitation of a ^{35}S methionine labelled 49kDa protein solubilised from B82 cells but no apparent immunoprecipitation of the human beta-2 receptor (Fig. 54).
11. Purified B4A8-PWL IgM monoclonal antibody at a concentration of 0.2g/L had no inhibitory effect on maximal isoproterenol stimulated cAMP release from Wt-7 Cape Town cells (See 6.5.2).

5.7 Production of a rabbit polyclonal antibody to beta receptor peptide: BR20II

Since it was clear that a monoclonal antibody raised to BR-20-II could at the most have a specificity for a single epitope on the peptide, a New Zealand White rabbit was immunised with the peptide in order to raise a polyclonal antibody which would have wider specificity and therefore better recognition. The effects of such an antibody on ligand binding are more likely to mimic those encountered in human sera, and I hoped to obtain an antibody which would in fact recognise the beta-2 receptor on Western blots or in functional assays.

1mg of BR-20-II in complete Freunds adjuvant was injected subcutaneously and the rabbit boosted after six weeks with incomplete Freunds adjuvant. The rabbit serum was harvested one week after boosting.

Recognition of the peptide BR-20-II by the polyclonal rabbit antibody was confirmed using an adapted peptide ELISA, in which the second antibody was a peroxidase-conjugated goat anti-rabbit antibody. The rabbit antibody had a titre of 1:400 in the ELISA. In a Western blot of human

foreskin fibroblast membranes and Wt-7 Cape Town membranes separated on a 9% PAGE under reducing conditions, the 49 kDa band was only recognised weakly, but the antibody also strongly recognised several other bands (64kDa, 59kDa and 22kDa (Fig. 56)). It is unlikely that the 64kDa or 59kDa bands represented binding to beta-2 receptors since they were also recognized in cells (B82) not transfected with the beta-2 receptor gene and as in Fig. 48, only 26ng of beta-2 receptor was loaded per lane.

In the [^{125}I]-CYP ligand binding assay a 1:10 dilution of the rabbit antibody produced 41% inhibition of ligand binding to membranes from high expressing 4000 fmol/mg beta-2 receptor transfected B82 cells, and 50% of ligand binding to low expressing cells (411 fmol/mg). Unimmunised rabbit serum inhibited ligand binding by less than 25%. Inhibition of ligand binding was apparently greater than that obtained with the monoclonal B4A8-PWL (see 5.6.7) and was of the same order as that produced with some of the sera from the asthmatic patients in Chapter 3. It is thus likely that this rabbit antibody did in fact possess anti-beta-2 receptor specificities. Unfortunately the initial rabbit developed a viral infection and died and two further rabbits were immunised with BR-20-II peptide. Antibodies raised in these rabbits Code 700 and 701 also recognised the peptide in the ELISA and were used for the studies on basal and maximal adenylate cyclase activation described in 6.5.2.

5.8 Cross immunogenicity of human beta-2 adrenergic receptors: immunization of C3H mice with intact human beta-2 receptors in mouse membranes.

B82-L cells are derived from C3H/AN mouse strain. Transfection and expression of the human beta-2 receptor into B82-L cells enabled me to conduct a completely unique experiment on the cross-species immunogenicity of intact human beta-2 receptors. Using this system the human beta-2 receptors are presented out of the context of other human antigens, in the C3H/AN mouse, without having to tediously affinity-purify human beta-2 receptors from its normal human context. I considered that documentation of cross-species immunogenicity of this highly conserved mammalian receptor could have possible implications for its immunogenicity in man.

Three C3H mice were immunized with 2400fmol beta-2 receptor in 800μg B82 membrane protein intraperitoneally in complete Freunds adjuvant and boosted again after 6 and 10 weeks with 2400fmol beta-2 receptor in incomplete Freunds adjuvant. The mice were bled from the retro-orbital plexus and the specificity of the antibody induced was investigated using Western blots of membranes from B82, human foreskin fibroblasts and red blood cells.

Strong bands of molecular weight between 60-68 000 daltons were found in both human fibroblasts and transfected B82 cells but not in the red cells suggesting initially that the antibodies were directed against the beta-2

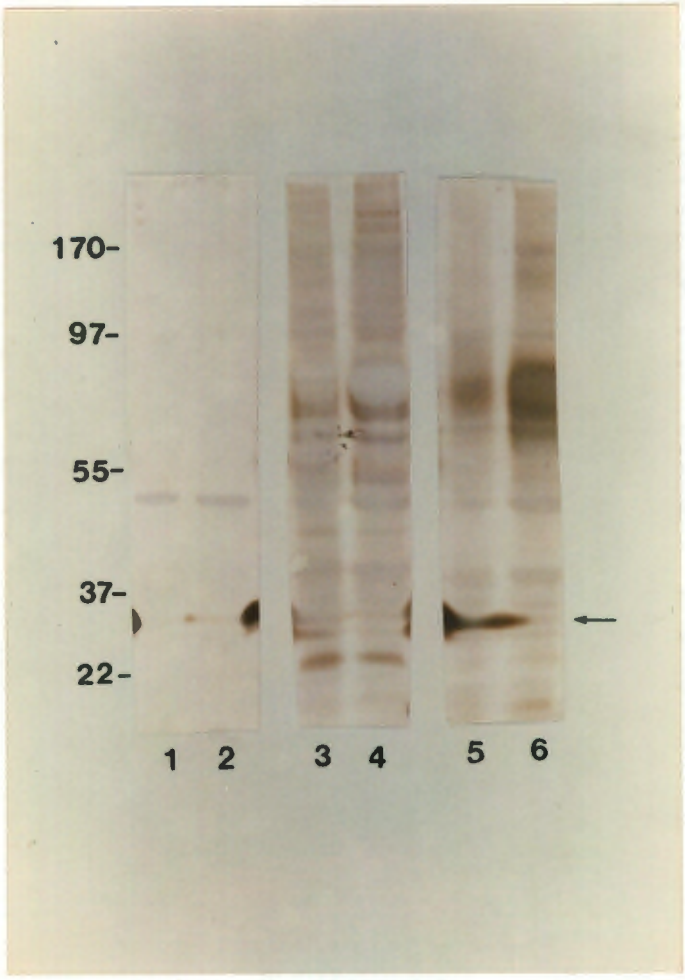


Figure 56. Western blot of 11% PAGE of human foreskin fibroblast membranes (lanes 1, 3, 5) and Wt-7 Cape Town membranes (lanes 2,4,6) incubated with 1:500 dilution of B4A8-PWL monoclonal antibody (lanes 1 & 2). 1:200 dilution of rabbit anti-peptide antibody (lanes 3 & 4) and 1:33 dilution of C3H mouse serum (immunised with Wt Cape Town membranes)(lanes 5 & 6). Note: Heavy blotchy staining at approx. 37kDa was artifact). Molecular weight markers (kDa) are shown on the left.

receptors, but when the studies were repeated, including membranes from untransfected B82 cells and transfected B82 cells with different levels of beta-2 receptor expression, no difference in the intensity of the 60-68 kDa bands was found (Fig. 57) with the B82 cells. These experiments suggested however that the intact transfected human beta-2 receptor was probably not immunogenic in the C3H mouse even with the addition of Freunds adjuvant. However the detection system for anti-beta receptor antibodies again may not have been sensitive enough. Dilutions of the mouse sera did not inhibit [^{125}I]-CYP binding more than sera from control unimmunised mice. No evidence of the development of any disease was found in the mice, 18 months after the immunization.

5.9 Discussion

Induction of antibodies to the human beta-2 adrenergic receptor in laboratory animals requires that certain conformational or sequential epitopes of the receptors are presented to the immune system of the immunised animal as "foreign".

In this chapter I have explored the immunogenicity of 20 amino acid hydrophobic peptide synthesised from a known sequence of the second transmembrane domain of the human beta-2 receptor. The immunogenicity of peptides derived from this domain has not previously been investigated. I have also investigated the immunogenicity of the intact human beta-2 receptor, presented to the mouse within a mouse membrane context. I was unable to show that the mouse monoclonal antibody raised to the peptide recognised the human beta-2 receptor. Similarly there was no evidence

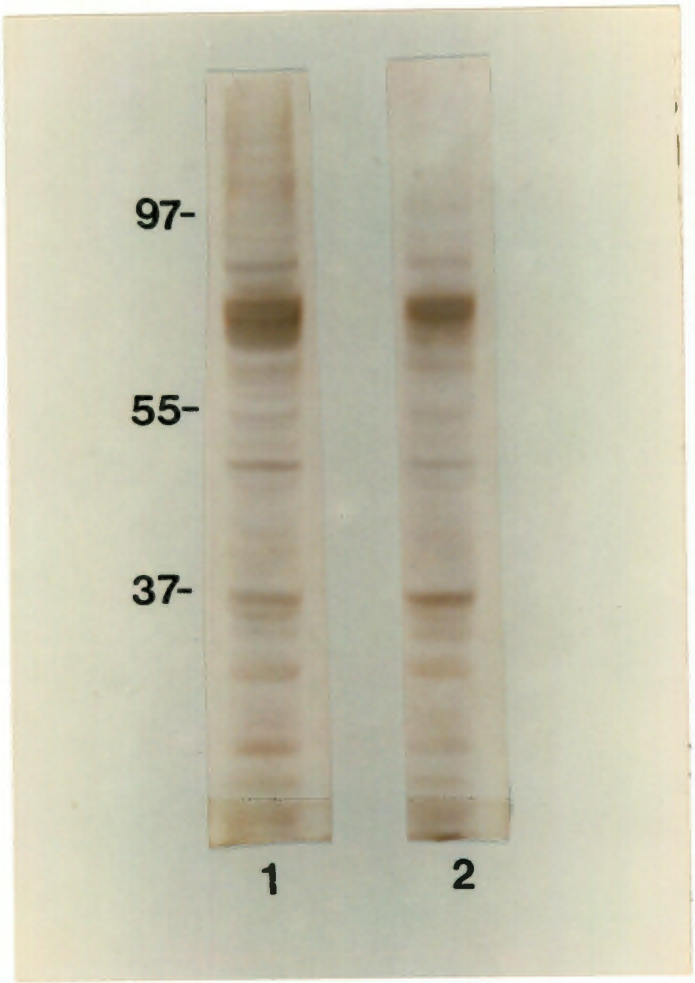


Figure 57. Western blot of 11% PAGE of untransfected B82 cells (lane 1) and transfected Wt-7 Cape Town cells (lane 2) and probed with 1:33 dilution of C3H mouse serum (immunised with Wt-7 Cape Town cell membranes).

that the intact human beta-2 adrenergic receptor was immunogenic in the C3H mouse.

There was also no direct evidence on the Western blots that the rabbit anti-peptide antibodies recognised the human beta-2 receptor but there was an increase in the [^{125}I]-CYP ligand inhibitory activity above control serum (1.5-2 fold), and paradoxically, the rabbit anti-peptide antibody also stimulated adenylate cyclase activity, i.e. displayed some "intrinsic sympathomimetic activity", which may or may not have been exerted at the ligand binding site.

The choice of the peptide derived from a single domain, of the human beta-2 receptor for production of these antibodies in mice and rabbits was influenced by the evidence supporting the role of this domain in ligand binding discussed in the introduction of this chapter, but ideally, all of the hydrophobic domains should be studied for immunogenicity, to produce a complete picture. More recently, Eshdat et al. (1989), showed that Iodocyanopindolol diazirine reacted with an amino acid residue which was located in a non-glycosylated region containing the sixth and seventh transmembrane domains of the receptor and that aspartic or glutamic acid residues were important for ligand interaction.

Clearly, at the time of selecting a peptide for a monoclonal antibody production conceptual models of ligand binding were not as complete as they are at the time of writing up this thesis.

The peptide chosen and synthesised from Asp 79 - Met 99 of the beta-2 receptor was extremely insoluble in aqueous solvent being composed of largely hydrophobic residues. Its inherent immunogenicity was unknown and two similar peptides were synthesized, also linked to cysteine residues (C and N-terminal), for possible coupling to keyhole limpet haemocyanin, if the peptide BR-20-II coupled to the palmitoyl residue failed to be immunogenic in the mouse. In view of the good response to the immunization of BALB/c mice with the palmitoyl peptide, the cysteine linked peptides BRP-20-I and BRP-20-III were not used for immunization, but it was useful having these peptides for the inhibition assays.

Except for initial difficulties in establishing a peptide ELISA relating to the insolubility of the peptide BR-20-II in aqueous solutions, production of a monoclonal antibody of IgM isotype which recognised the peptide to a titre in 1:48 000 in the ELISA was uncomplicated. Inhibition experiments (5.6.2) confirmed that the antibody recognised the free (probably aggregated) peptide as well as the peptide attached to the plastic (Titertek).

The monoclonal B4A8-PWL demonstrated no apparent recognition of glycosylated species of beta-2 receptors (MW 65-75kDa) on the Western blots of transfected L cells expressing high levels (4000fmol/mg) of beta-2 receptors (26ng per lane). It is possible that the amount of receptor protein may have been below the limits of detection for the immunoblot. There was no doubt that the monoclonal recognised a 49kDa protein, present on both transfected and non-transfected B82 mouse cells (5.6.4). Immunofluorescence studies (5.6.5) indicated that the 49kDa

protein was present in an intramembranous or intracellular location since positive fluorescence was only obtained with fixed (permeabilised) cells.

Since the reported size of the unglycosylated human beta-2 receptor is 46 480 daltons, it was important to be sure that the 49 kDa protein did not represent a partially processed beta-2 receptor. Although it was reported that the B82 cells do not express functional beta-2 receptor (Gilman, 1973) the mechanism for the failure to express beta-2 receptors has not previously been investigated. There seemed to be several possibilities: either the gene for the beta-2 receptor or its regulatory sequences have been deleted, or the B82 cells had acquired transcriptional or translational defects. Probing Southern blots of DNA from both the transfected and non-transfected cells with 2.6kb beta-2 receptor gene probe, revealed total absence of beta-2 receptor gene sequences in the non-transfected cells. These autoradiographs demonstrate for the first time, to my knowledge, that the B82 cells in fact have a deletion of the beta-2 receptor gene.

Based on the above experiment, it was quite clear that the 49kDa band detected by the monoclonal B4A8 PWL in the non-transfected cells, could not be partially processed beta-2 receptor. However, a relationship between the 49kDa protein to the beta receptor sequence in the peptide Asp^{79}-Met^{99} does exist, since the monoclonal B4A8-PWL clearly recognises both. Since sequence homology between beta-2 receptor and other members of the seven transmembrane supergene family is well recognized, the 49 kDa protein could represent a related or new member of this family of intramembranous proteins. The G21 protein (42 000Mr) identified by

Kobilka (1987) also shares 43% homology with the human beta-2 adrenergic receptor in the transmembrane domains. RNA for the G-21 protein was found to be widely distributed (lymph nodes, gut, muscle). However, if the B82 cells have deleted the beta-2 receptor gene by chromosomal loss then they might also have deleted G-21, if both genes share the same chromosome as they do in humans (Chromosome 5).

Definitive identification of the 49kDa protein would require affinity purification of the protein and either complete amino acid sequencing or partial sequencing of a peptide of the protein and construction of an oligonucleotide probe which could be used to screen a fibroblast cDNA library to sequence the DNA. It may also be possible to screen the library with the monoclonal B4A8-PWL. This investigation however is beyond the aims of this thesis but would be important to do in the future.

I was unable to demonstrate that the monoclonal B4A8-PWL recognized solubilised intact beta receptors (removed from their membrane constraints) but the antibody did display some biological activity by inhibiting cell proliferation, (5.6.7) probably through its interaction with the 49kDa protein.

[^{125}I]-CYP binding inhibition assays conducted with varying dilutions of the monoclonal B4A8-PWL and with an irrelevant monoclonal (αIgE) were also informative. Firstly, similar low degree of inhibition (<30%) of ligand binding was obtained with both antibodies, and secondly, the degree of inhibition was greater when membranes from B82 cells expressing lower levels of beta-2 receptors were used. Since the two monoclonals

have very different specificities, the inhibitory effect found in these assays would appear to be non-specific, possibly due to interactions of non-variable domains of immunoglobulins with the receptor, or due to other factors in the dilutions of the ascites fluids or harvest fluids.

These findings cast new light on the inhibition assays performed with human sera reported in Chapter 3.2 since, in addition to any possible specific inhibitory effects which one could attribute to the immunoglobulin fraction, mouse ascites also possess other non-immunoglobulin factors which partially prevent beta-2 receptor antagonists from binding to their receptors.

Inability to produce a monoclonal antibody which could recognise the intact human beta-2 receptor, the Western blotted receptor, or significantly inhibit ligand binding, could relate to differences in three-dimensional configurations of the peptide BR-II-20 versus the intact receptor and the beta-2 receptors once subjected to SDS-PAGE linearised and Western blotted. It is known (van Regenmortel - personal communication) that even for a 20 amino acid peptide, many conformational forms are possible and consequently a wide range in specificities of the resulting monoclonals raised to the peptide are possible, and one may have to perform several fusions and many screenings to obtain a monoclonal antibody which recognises the epitope in the intact molecule. Van Regenmortel has also stated that for such studies, polyclonal antibody production may be more useful.

Several groups have now produced polyclonal antibodies to peptides derived from the human beta-2 adrenergic receptor (Table 15)(Aoki et al. 1989, Magnussen et al. 1989, Dunkel et al. 1989, Theveniau et al. 1989 and Wang et al. 1989). The rabbit polyclonal antibody I raised to the BR-20-II peptide recognised a number of protein bands on the gel (Fig. 56) including weak recognition of the 49kDa protein. This antibody also exerted stimulatory effects on basal adenylate cyclase activity. These effects could have been exerted at a non-ligand binding site, since I did not demonstrate direct binding to the human beta-2 receptor. I have not attempted to immunoprecipitate the solubilised human beta-2 receptor, using this antibody, but this should be done. The rabbit antibody produced similar degrees of inhibition of [^{125}I]-CYP binding as many of the normal human sera exerted (3.2). Since stimulatory activity on basal adenylate cyclase activity was increased by further immunization with the peptide (6.5.2) one of the conformational epitopes recognised by the rabbit polyclonal antibody (701) may have been the beta-2 receptor. To confirm this it would be important to study the effect on adenylate cyclase activity in both transfected and non-transfected B82 cells.

Polyclonal anti-peptide antibodies to 11 hydrophilic peptide sequences of the hamster beta-2 adrenergic receptor have been successfully raised by Wang (1989) but there are as yet no published reports of any attempts to raise antibodies to any of the hydrophobic intramembranous domains, with which to compare my own data. The studies by Wang supported the predictive value of hydropathy analysis for the domains which do not have a membrane spanning location. It is relevant to note that in their studies the 11 peptides varied in their immunogenicity in the rabbit and

that with one of the peptides (CM-10 - N terminal peptide) polyclonal antibodies were only obtained after immunization of 7 animals.

Polyclonal antibodies raised to peptides derived from the second extracellular loops of the human beta-1 and beta-2 receptor by Magnusson et al. (1989) could be used immunohistochemically to visualise the beta adrenergic receptors on rabbit heart. The anti-beta-2 peptide antibodies did not show any functional effect on the beta adrenergic receptors but the anti-beta-1 peptide antibodies were able to displace agonist affinity to higher values. The studies are of particular interest since they identified possible T and B cell epitopes in the second extracellular loop which induce an immune response and they suggested that extracellular epitopes such as these may be important in the induction of autoimmune responses *in vivo*.

The monoclonal anti-peptide antibody produce by Aoki et al. 1989 has been used to localise beta receptors in neurones and dentrites. I have summarised the main functional characteristics of important published studies of antibodies raised to beta adrenergic receptors, or to peptides derived from beta receptors in Table 15.

Table 15 also illustrates that successful antibody production to beta adrenergic receptors is possible using partially purified adrenergic receptors (Fraser and Venter 1980), affinity chromatography purified receptors (Courad et al. 1981, Strader et al. 1983, Moxham et al. 1986) beta receptor ligands (Strosberg et al. 1985, Guillet et al. 1984) or

TABLE 15: FUNCTIONAL CHARACTERISTICS OF PUBLISHED ANTIBODIES RAISED TO BETA ADRENERGIC RECEPTORS

Receptor antigen used in immunization	Species immunised and type of antibody studied	Main Functional Characteristics	Reference
Partially purified turkey erythrocyte B1	BALB/c mouse monoclonal ascites - 104	Competitive inhibition of [^{125}I]-HYP binding to turkey erythrocytes. Immunoprecipitation of partially purified beta-1 receptors. Affinity (estimated) 0.2-1μM	Fraser C. and Venter J.C. 1980
Partially purified turkey erythrocyte B1	BALB/c mouse monoclonal-103	Non-competitive inhibition of [^{125}I]-HYP ligand binding to turkey erythrocyte B-receptors. Immunoprecipitation of partially purified turkey erythrocyte B1 receptors	Fraser C. & Venter J.C. 1980
Turkey erythrocyte B1(affinity chromatography purified)	Mouse polyclonal (Protein A Sepharose affinity purified IgG)	Immunoprecipitation of radiolabelled purified beta-1 receptor. Stimulation of adenylate cyclase in turkey erythrocyte membranes.	Courad P.O. et al. 1981
Frog erythrocytes B2 (affinity chromatography purified)	Rabbit polyclonal $(NH_4)_2SO_4$ precipitate	Immunoprecipation of purified frog erythrocyte beta-2 receptors. No effect on antagonist binding. Selective attenuation of isoproterenol stimulated adenylate cyclase activity. Post-synaptic localization of beta receptors in rat and frog brain. No cross reactivity with beta-1 receptors	Stader C. et al.1983
Class 1 alloimmune	Mouse IgG	Binding to beta receptors of mouse myocardium. Competitive inhibition of [^{3}H]-DHA binding to mouse myocardial membranes. Induction of positive inotropic and chronotropic effects on isolated mouse atria	Sterin-Borda et al. 1984

Receptor antigen used in immunization	Species immunised and type of antibody studied	Main Functional Characteristics	Reference
Monoclonal anti-beta adrenergic ligand	Monoclonal anti-idiotypic	Recognition of the monoclonal idiotype. Inhibition of hapten binding (6 clones) Recognition of beta adrenergic receptors (3 clones)	Guillet et al. 1984
CFI sera	BALB/c IgG	Sustained inhibition of spontaneous oviduct motility during the whole sex cycle. Blocked by 10^{-7} L(-)propranalol. Effectiveness of immune IgG depenent on varying affinity of beta-adrenoceptors during cycle.	Borda E.S. et al. 1984
Anti reovirus antibody	Monoclonal and polyclonal anti-idiotypic	Immunoprecipitation of purified beta adrenergic receptors	Co et al. 1985
Frog erythrocyte plasma membrane digitonin extract	Mouse monoclonal	Immunoprecipitation of a solubilised beta adreneric receptor labelled with [^{125}I]-iodohydroxybenzyl pindolol	
		Activation of adenylate cyclase in activated erythrocyte plasma membranes	Chuang 1985
		Interaction with 43000 Mr protein with a pI = 6.2	

Receptor antigen used in immunization	Species immunised and type of antibody studied	Main Functional Characteristics	Reference
Monoclonal anti-alprenalol antibody	Mouse monoclonal anti idiotypic	Stimulation of catecholamine sensitive adenylate cyclase blocked by propranalol	Strosberg A.D. et al 1985
Guinea pig lung B2 (affinity chromatography purified)	Rabbit polyclonal	Inhibition of ligand binding [^{125}I]-CYP to S49 mouse lymphoma beta-2 receptors. Immunoprecipitation of beta-1 (fat cell) receptors	Moxham C. et al. 1986
Rat fat cell B1 (affinity chromatography purified receptor)	BALB/c mouse polyclonal	Binding to 5ng/well immobilised rat fat cell purified beta-1 receptor in ELISA. Inhibition of ligand binding [^{125}I]-CYP (non competitive)(no change in affinity of ligand) to rat fat cells. Immunoprecipitation of beta-2 receptors (rat liver)	Moxham C. et al. 1986
Purified turkey erythrocyte beta-1 receptor	15 mouse monoclonals	Immunoprecipitation of solubilised Turkey beta-1 receptors and cross reactivity with with beta-2 receptors. One antibody inhibited adenylate cyclase activity.	Itami et al. 1986
Affinity purified beta receptor from A431 cells	BALB/c mouse monoclonals IgM, IgG2a and IgG3	Positive recognition of beta-2 receptors by immunofluorescence, immunoprecipitation and on immunoblot	Kaveri et al. 1987

Receptor antigen used in immunization	Species immunised and type of antibody studied	Main Functional Characteristics	Reference
Beta receptor peptide aa 226-239	Monoclonal	Localization of beta receptors in neurones and proximal dendrites	Aoki et al. 1989
Turkey Beta 1 N terminus (aa 1-10)	Polyclonal	Recognition of the 50kDa but not the 40kDa form of the receptor	Dunkel F.G. et al. 1989
Rodent lung beta-2 adrenergic receptor	Polyclonal	Punctate immunofluorescence of fixed intact cells	Wang et al. 1989
Affinity purified beta-1 receptor (Turkey)	Mouse monoclonal	Recognition of 42kDa protein on nitrocellulose. No inhibition of ligand binding. Also effect on adenylate cyclase	Chapot et al. 1989
Extracellular loop of beta-2 receptor aa 16-31 aa 174-189	Rabbit polyclonal	Recognition of purified beta-2 adrenergic receptor on immunoblots	Theveniau et al. 1989
11 hybrophilic peptides of beta-2	Rabbit polyclonal	Recognition of 65kDa protein on immunoblots and positive immunofluorescence on intact and permeabilised cells	Wang et al. 1989
C terminus transfer beta receptor	Mouse monoclonal G27	Selective immunoprecipitation of rodent beta-2 receptors	Romano et al. 1989

synthetic peptides of the beta receptor (Aoki et al. 1989, Wang et al. 1989).

These antibodies have demonstrated different specific applications e.g. inhibition of ligand binding, immunofluorescence, immunoprecipitation and some have demonstrated functional effects on adenylate cyclase activity (Courad et al. 1981, Strosberg et al. 1985, Sterin Borda et al. 1984).

The immunogenicity of the intact human beta-2 receptor has not to my knowledge been previously investigated in the mouse. The immunization protocol described in 5.8 did not induce antibodies to the human beta-2 adrenergic receptor that I could detect. It would have been most useful if I could have had a source of affinity-purified beta receptors with which to test my own rabbit and mouse antibodies, but I have obtained insufficient yields of beta receptors when I performed alprenalol affinity chromatography, for such studies.

All of the above studies, including my own have investigated the immunogenicity of intact beta receptors or synthetic peptides derived from beta receptors using "non-physiological" systems. It would seem that there are no animal models which demonstrate spontaneous development of autoantibodies to the beta-2 adrenergic receptor. Moxham et al. (1986) has remarked that in their experience beta-1 receptors purified from S49 mouse lymphoma cells were found to be poor immunogens in the rabbit. In none of the above studies in Table 15 has it been reported that the induction of antibodies to beta adrenergic receptors led to any autoimmune process in the immunised animals, and in particular, there is

no evidence that any of these animals developed bronchospasm, or hypotension or cardiomyopathy.

The case for a functional role for antibodies to beta-1 receptors in disease is gaining acceptance. Since the evidence for the development of autoantibodies in humans to beta-1 adrenergic receptors has been published (Borda et al. 1984, Sterin Borda et al. 1984) it has been shown that these antibodies may have functional effects on adenylate cyclase (Limas et al. 1990, Gorelik et al. 1990) and that their induction may be linked to either HLA DR4 or HLA DR1. It has also been shown very recently (Cremaschi et al. 1990) that antibodies to H2 Class I histocompatibility molecules were capable of inhibiting specific beta adrenoceptor ligand binding to purified cardiac and smooth muscle membranes and simultaneously could behave as beta adrenoceptor agonists.

The findings of Cremaschi et al. (1990) are not unlike my own findings with the rabbit polyclonal (701) antibody which displayed inhibition of antagonist binding but had a stimulatory effect on basal adenylate cyclase activity. While evidence for autoantibodies to cardiac beta-1 receptors is increasing, evidence for a functional role for autoantibodies to the human beta-2 receptor remains scant (Fraser 1984, Wallerkat et al. 1987, Blecher et al. 1984).

Autoantibodies to beta-2 receptors could "spontaneously" arise, following a viral infection as part of an anti-idiotypic response (Plotz 1983) and in this context anti-reovirus antibodies are of interest (Williams et al. 1989). Liu et al. (1988) have shown that the reovirus 3 and [^{125}I]-CYP

bind to distinct domains of the beta adrenergic-like receptor. Anti-idiotypic antibodies to the reovirus could thus theoretically bind to beta-2 receptors and influence ligand binding non-competitively.

Autoantibodies to hormone receptors may also arise in the context of aberrant HLA DR expression as suggested by Hanifuzo et al. (1983) and Botazzo et al. (1983) in thyroid disease. Recent studies by S. Holgate, UK (unpublished and presented at the S.A. Pulmonology Congress, March, 1990) have shown that there is indeed an increase in Class II expression in cells lining the airways of asthmatic subjects. These studies may have an important bearing on mechanisms of induction of anti-beta-2 receptor antibodies in asthmatics and studies of the relationship between HLA class II expression in the airways and [^{125}I]-CYP inhibitory activity in the serum are awaited.

CHAPTER 6

STUDIES OF CLONED HUMAN BETA-2 ADRENERGIC RECEPTORS EXPRESSED IN L CELLS.

6.1 Introduction

Studies of intact cells permit receptors to be probed in their native environment where they are subject to regulation by intracellular events, organelles and constituents. Although certain properties are quite similar in membranes and intact cells, binding characteristics vary significantly. In this context, Insel (1983) reported a striking discrepancy between the Kd of an agonist determined in competitive assays with a radiolabelled antagonist and its Kact, (found to be two orders of magnitude less than the Kd).

The data I have presented in Chapter 3, using the beta receptor antagonist [^{125}I]-iodocyanopindolol and the guinea pig lung membrane preparation, confirmed that human serum *in vitro* does in fact modulate antagonist binding to guinea pig lung beta adrenergic receptors in membrane preparations, but also raised a number of intriguing questions relating to the possible physiological or pathophysiological significance of this phenomenon, if any, in intact cells.

In the first instance, it was important to demonstrate that this is not simply a cross-species phenomenon observed with calf lung or guinea pig lung membranes and that human serum can indeed influence

ligand binding to human beta-2 receptors. It is also important to know whether human serum also modulates agonist activated human beta-2 receptor function, before a pathophysiological role for the serum factors can be suggested. Since it is likely that, by the nature of its preparation, the membranes isolated in Chapter 3 from whole organ homogenates do not represent a physiological situation, I considered it important to study the phenomenon in viable cells under less harsh conditions than those used in the membrane preparation.

Although the adenylate cyclase assay of Salomon (1974) used in the studies of Chapter 2 was sensitive and reliable for studies for homogenates, I did not consider it suitable for studies of the effects of whole or diluted serum in adenylate cyclase, since diluting the incubation mixture with serum is likely to induce further artefacts, making interpretation of the results difficult. Tissue homogenates are also unphysiological assay systems and an *in vitro* system utilising homogenates and membrane preparations for studies of functional effects of serum on beta-2 receptors certainly does not have an *in vivo* counterpart. It is not possible to study the effects of serum directly on cells *in vivo*, and studies of the modulating effects of serum on cloned human beta-2 receptors in cells in culture have not been previously reported.

Following the cloning and sequencing of the human beta-2 adrenergic receptor by Chung et al. (1987) and in view of my previous experience with transfection of both wild type and mutant beta-2 receptors into B82 mouse fibroblasts at the N.I.H. (Chapter 4), on my return to Cape Town I considered that it would be worthwhile to conduct further

transfections of the wild type beta-2 receptor gene using the plasmid pMSV Neo in order to obtain a line of cells stably expressing the human beta-2 receptor at a higher level than previously reported (Fraser et al. 1987). Such a line could be used as a rich source of human beta-2 receptors for Western blots and antibody studies using immunofluorescence, and could be used to study the effects of human serum fractions on beta-2 receptor mediated adenylate cyclase activity in whole cells.

I have successfully transfected the human beta-2 receptor gene into the B82 line of L cells and have found that certain human sera markedly inhibited adenylate cyclase activity. However, the nature of the inhibition was unexpected. In this chapter I have described the transfections I have performed to obtain a line of cells expressing human beta-2 receptors at high level, the experiments I have conducted to investigate the effects of human sera and serum factors on adenylate cyclase activity and the limitations of this system for studies with human serum. I have also used these transfected cells as a source of human beta-2 receptors for follow-up studies of the serum inhibitory effects on membrane preparations (described in Chapter 3) and to study the effects of purified immunoglobulins and cellular fractions on beta-2 receptor function.

6.2 High level transfection of human beta-2 adrenergic receptors and reconstitution of hormone sensitive adenylate cyclase in mouse (B82) L cells.

6.2.1 High level transfection of human beta-2 receptors into mouse (B82) L cells.

The beta-2 receptor gene cloned into the new expression vector pMSV Neo was used for the transfections. This expression vector had been constructed at the N.I.H. by Dr. Fuzon Chung by replacement of the xanthine guanine phosphoribosyltransferase gene in plasmid pMSG with the neomycin-resistance gene and the construction of a new polylinker to facilitate cloning of the beta-2 adrenergic receptor gene. For receptor expression the entire coding region of the human beta-2 adrenergic receptor derived from the genomic clone LCV-518 (Chung 1987) (base pairs - 6 to 1267) was cloned into the Mlu and Eco R V sites in the polylinker region of pMSV neo. This expression vector pMSV neo contains the 1450 base pair long terminal repeat (LTR) of the mouse mammary tumour virus (MMTV) and the dexamethazone inducible transcription promoter within the MMTV 5' long terminal repeat regulates expression of the genes inserted into the downstream multiple cloning site. The neomycin resistance gene is expressed from an SV40 early promoter to ensure selection of stably transformed cells when grown in medium containing the Neomycin analogue Geneticin (G418). TG-1 E Coli were transformed with the pMSG Neo expression vector and grown up overnight followed by a plasmid preparation using the method of Davis et al. (1986). After further purification by CsCl

centrifugation, and precipitation in ethanol overnight, at -70°C, to sterilise the expression vector, 100μg DNA (pMSV neo) was used for the transfection.

On the day before the transfection, B82 cells grown to confluency in 10cm dishes were split 1:5 and plated out in RP-10 (RPMI containing 10% foetal calf serum). On the day of transfection the DNA was air dried in a laminar flow tissue culture hood (Laminaire, Bino Instrumentation, Sanlamhof) prior to resuspending it in 450μl sterile H_2O and adding 50μl of 2.5M $CaCl_2$. B82 cells (see Appendix E) were fed with fresh RP-10 and allowed to settle for 4 hours. One hour before the transfection, the DNA, in calcium chloride (550μl), was added dropwise to 500μl 2x Hepes buffered saline (HeBS) pH 7.05 while bubbling air through a mechanical pipettor, vortexed and the precipitate allowed to stand for 20 minutes at room temperature.

Using a Pasteur pipette the precipitate was distributed evenly over the 10cm dish and after the dish was agitated, the cells were incubated at 37°C for 4 hours. The medium was then removed and 2.0ml of sterile 10% glycerol solution was added for 3 minutes at room temperature after which 5.0ml of PBS was added, the plate agitated and supernatant removed. After washing twice with PBS the cells were fed with RP-10 (without geneticin). After 2 days, geneticin G418 sulphate (Gibco), in a final concentration of 500μg/ml, was added and the cells allowed to grow for one week. Four geneticin resistant clones were chosen, re-plated, and pulsed with 10^{-6}M dexamethazone before screening the cell membranes of the positive clones (Appendix F) for beta-2 receptor expression using a [^{125}I] cyanopindolol binding assay.

On initial screening all four clones expressed human beta-2 receptors ranging from 200fmol/mg to 3200fmol/mg membranes (Kd [^{125}I]-CYP 20-37pM).

I also used the above protocol to transfect the serine 346 mutant beta-2 adrenergic receptor into the B82 cells (see 4.3.2).

6.2.2 High level stable human beta-2 adrenergic receptor expression in clone B82-Wt-7, Cape Town.

In the initial [^{125}I]-CYP binding assays, clone B82-Wt-7 had the highest level of beta-2 receptor expression and on subsequent plating, freezing, thawing, re-culturing and pulsing with dexamathazone (Sigma) the cells have stably expressed beta-2 receptors at levels of between 4000fmol-6000fmol/mg membrane protein during the eighteen month period of these studies. A saturation curve and Scatchard plot of the binding of [^{125}I]-CYP to clone Wt-7 is shown in Figure 58. Clone Wt-7 was used for most of the functional studies reported in this chapter. This new high expressing clone I have designated Wt-7 Cape Town. It is of interest that with this clone, beta-2 receptors were constitutively expressed (at approximately 400fmol/mg protein) even in the absence of dexamethazone pulsing. Constitutive expression is shown in the Scatchard insert in Fig. 58 (designated: B82 (low)). A photograph of Wt-7 Cape Town cells is shown in Fig. 58(a).

To my knowledge this level of stable expression of human beta-2 receptors in a cell line has not previously been achieved in

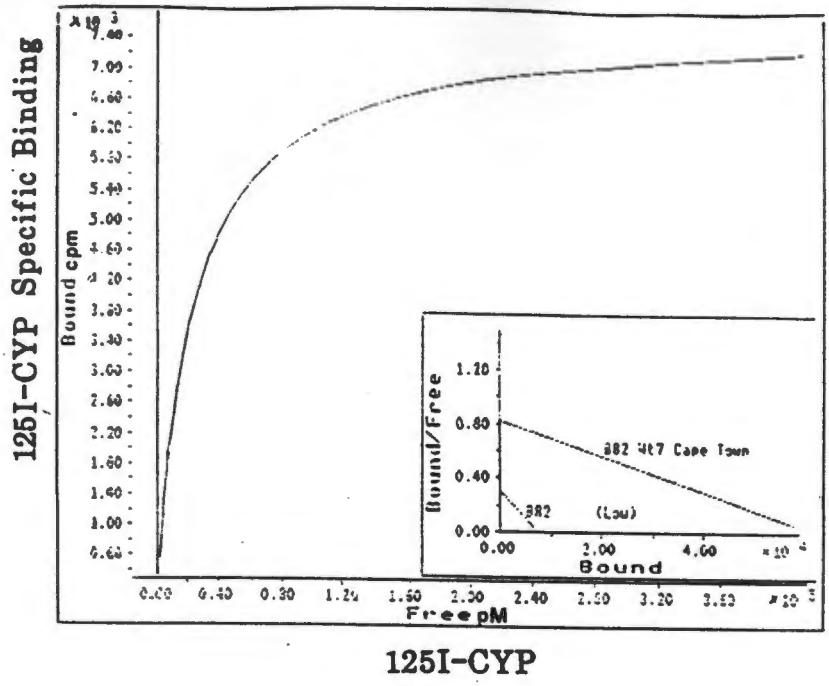


Figure 58. Saturation curve and Scatchard plot (insert) of [^{125}I]-CYP binding to Wt-7 Cape Town cell membranes 95μg) after stimulating for 16 hours with 10^{-6}M dexamethazone (Bmax 4400fmol/mg, Kd = 13 pM). Scatchard plot of constitutive expression of human beta-2 receptors (~400fmol/mg) in unstimulated cells is designated B82 (low).

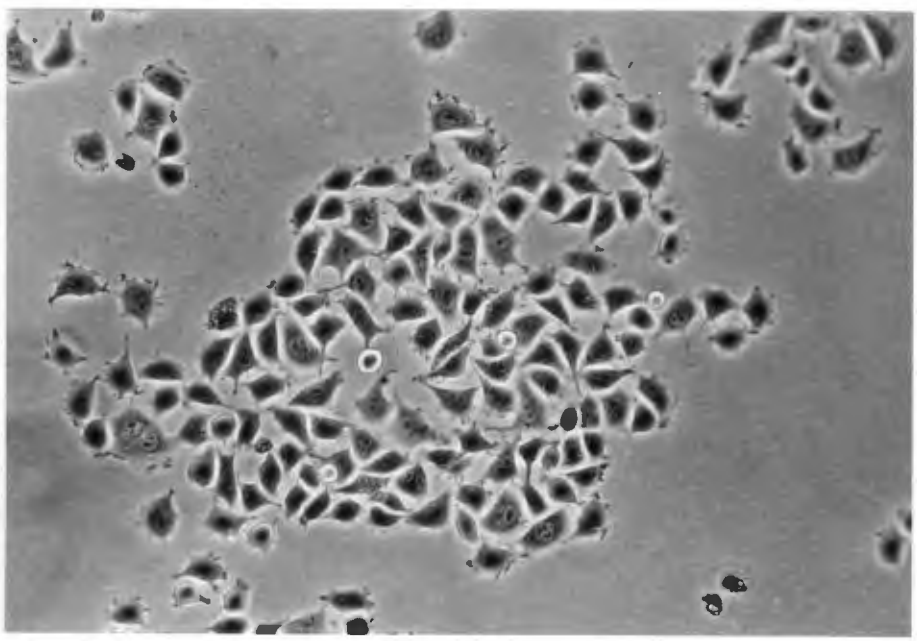


Figure 58a Photograph of Wt-7 Cape Town cells in culture (B82 fibroblasts transfected with the human beta-2 adrenergic receptor gene).

transfection experiments and thus clone Wt-7 Cape Town is a valuable source of human beta-2 receptors for further immunological,

biochemical and pharmacological studies. Bouvier et al. (1989) attained beta-2 receptor levels of 1200fmol/mg membrane protein in Chinese hamster fibroblasts (CHW 1102 cells)). Three other clones which I transfected, expressing lower numbers of beta-2 receptors were used to study the effect of receptor number in the serum inhibitory experiments (6.6.1). Polyacrylamide gel electrophoresis and Coomassie blue staining revealed no new visible protein bands in membranes of the transfected cells. A Southern blot of genomic DNA, digested with Pst 1, from transfected and non-transfected cells was probed with the nick translated cDNA 2.6 kb genomic probe (4.2.3). An autoradiograph of the blot shown in Fig. 51 confirmed an absence of the beta-2 receptor gene in non-transfected cells and gave a strong positive signal with the beta-2 receptor probe in the transfected cells. Constitutive expression of human beta-2 receptors had an inhibitory effect on growth of the cells in culture. Growth curves of the non-transfected B82 cells, "low" expressing 400fmol/mg B82 cells and "high" beta-2 receptor expressing B82 cells are shown in Fig. 59. (In the presence of 10^{-6}M dexamethazone, and induction of beta-2 receptor expression, there was a further reduction (20-30%) in the rate of cell growth observed at 24-48 hrs.)

6.2.3 Measurement of intra- and extra-cellular cyclic AMP.

For the studies of the modulating effects of human serum and cellular fractions on the function of human beta-2 receptors transfected into

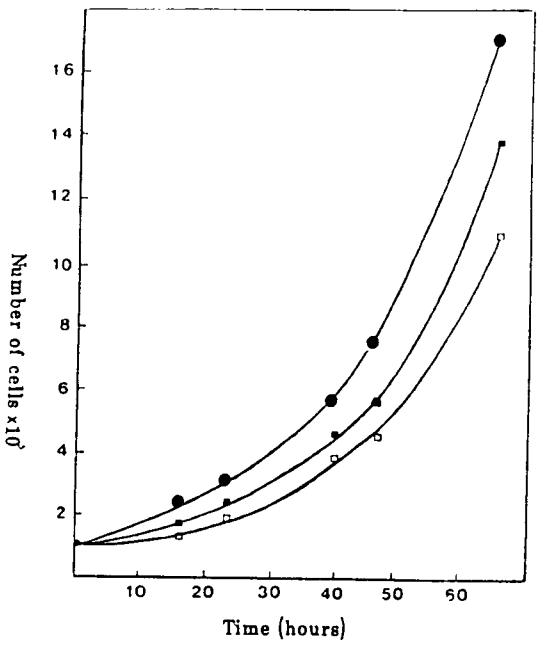


Figure 59. Growth in culture of untransfected B82 cells (●), B82 cells constitutively expressing low levels (■) and higher levels (□) of human beta-2 adrenergic receptors (in the absence of 10^{-6}M dexamethazone). $1x10^5$ cells from each cell type were plated (in duplicate) and the number of viable cells counted at 5 time points (16 hrs, 23 hrs, 40 hrs, 47 hrs and 65 hrs).

the Wt-7 Cape Town cells, the RIANEN (R) cAMP [^{125}]RIA kit (New England Nuclear) was used to measure cyclic AMP produced in the cells. These kits were found to be extremely reliable and reproducible, but an important limitation in my studies, was the cost of these kits which was amplified by the depression in the South African economy and poor Rand exchange rate.

Samples were prepared for the cyclic AMP assay as follows:-
24 hours prior to stimulating the cells, B82 or Wt-7 Cape Town cells plated in 6 cm (5ml) dishes were pulsed with 10^{-6}M dexamethazone (Sigma). Cells were used at half confluency (5 x 10^4 cells/plate). Kashles and Levitski (1987) have reported that confluency of cells in culture has an inhibitory effect on cAMP stimulation and have stressed the importance of conducting experiments of this nature with pre- or semi-confluent cells.

At the start of the assay the medium was removed, cells were washed twice in PBS and then pre-incubated with 1 ml PBS with 1mM isobutyl-methylxanthine (IBMX) (Sigma) (to inhibit phosphodiesterase activity) and dilutions of serum, immunoglobulin, supernatant or buffer for 30 minutes. To this was added concentrations of isoproterenol, prostaglandin E_1 (or other agonists) for 20 min, after which the supernatant (containing the extracellular cAMP) was removed and lyophilised. 1 ml of trichloroacetate was then added to the cells (containing intracellular cAMP) on each plate for 1-2 minutes and then extracted four times with 4 ml of water-saturated ether, prior to snap freezing and lyophilization. Lyophilised extracts of the extracellular and intracellular cAMP were pooled and reconstituted to

a final concentration of 10-25μg/100μl in PBS for the cAMP assay. For all of the studies reported in this chapter, total cAMP (extracellular cAMP plus intracellular cAMP) was measured and expressed in pmol/mg/20 min. Mean values and individual values obtained in duplicate experiments are shown on the bar graphs in this chapter.

Protein concentration of the cells on the plates was determined by the Biuret assay after adding 1ml of 0.2N NaOH to representative plates for 30 min to dissolve the protein. A standard curve for cAMP was run, and calculation of total cAMP (pmol/mg protein/20min) produced was measured exactly according to the radioimmunoassay protocol, described in the manufacturer's instructions of the RIANEN ™ cAMP radioimmunoassay kit (Du Pont). The standard curve of cAMP using this kit was highly reproducible from assay to assay.

6.2.4 B82 cells after transfection with the human beta-2 receptor gene couple with adenylate cyclase.

It was previously known that B82 cells possess prostaglandin E_1 receptors which couple with adenylate cyclase (Chlapowski et al. 1975). In order to confirm that the human beta-2 receptor in B82 transfected cells coupled with the mouse adenylate cyclase, cyclic AMP production (pmol/mg/20 min) was compared in the transfected and non-transfected cells following stimulation with 10^{-6}M (-)isoproterenol (Sigma) and 10^{-6}M prostaglandin E_1 (Sigma). Results of a duplicate experiment given in Fig. 60 show that transfection of the cells was accompanied by a marked increase in 10^{-6}M (-)isoproterenol stimulation. Basal levels of cAMP were higher in the transfected cells (± 1000 pmol/mg/20 min) compared with non-transfected

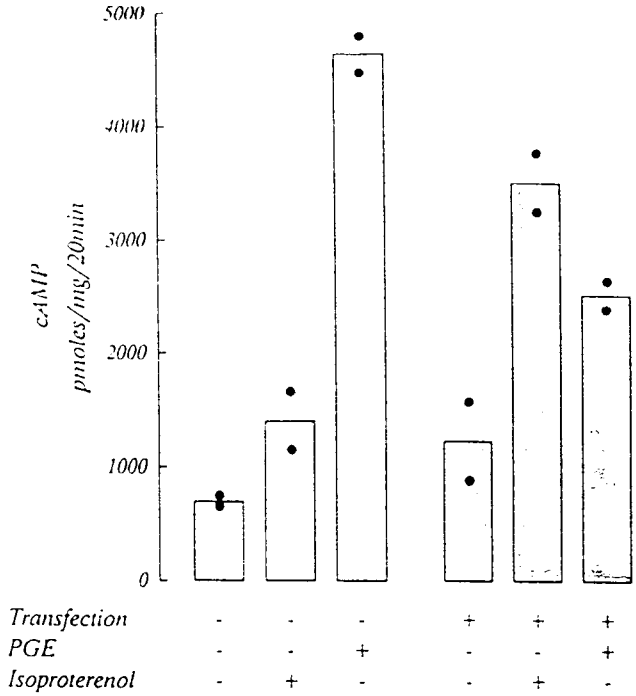


Figure 60. Basal, isoproterenol (10^{-6}M) and Prostaglandin E_1 (10^{-6}M) stimulated cAMP production in untransfected B82 cells (□) and Wt-7 Cape Town cells. (■) Bars indicate mean values of duplicate experiments (•).

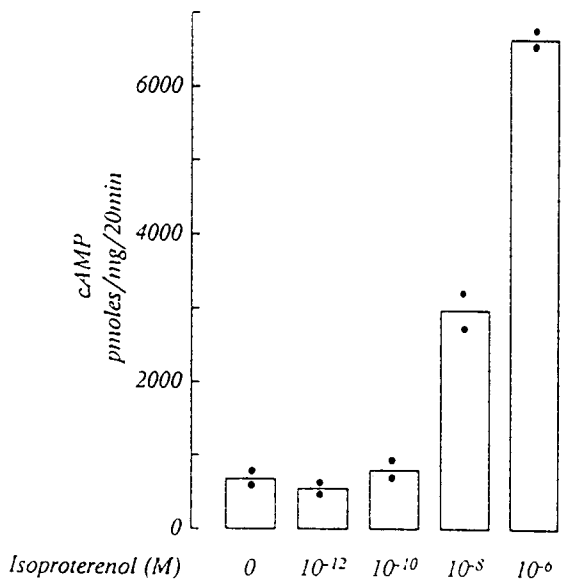


Figure 61. Dose response of (-) isoproterenol stimulation of cAMP production in Wt-7 Cape Town cells. Bars indicate mean values of duplicate experiments (•).

cells. Basal levels also varied with the level of beta receptor expression induced by 10^{-6}M dexamethazone in the transfected cells. A dose response of isoproterenol stimulation of the transfected cells is given in Fig. 61. In studies with Chinese Hamster cells transfected with human beta-2 adrenergic receptors, Bouvier et al. (1989) showed that a reduction (by alkalization) of up to 60% of receptors was necessary before reduction in the maximal isoproterenol stimulation was observed. It was therefore important to compare maximum isoproterenol stimulated cAMP production in clones transfected with different numbers of beta-2 receptors. A comparison of maximal cAMP release in a low expressing clone (480 fmol/mg) with a high expressing clone (4800 fmol/mg) given in Fig. 62 demonstrates that a 10-fold reduction in receptor number only reduced the total cAMP production by 50%. These data suggested that the excess receptors expressed are functionally redundant and that a dramatic reduction or inhibition of receptors is necessary before decreased in cAMP production are observed in the high expressing clones. I studied the kinetics of the cAMP release over time by stopping the reaction after 1, 2, 1, 20 and 30 min and found that maximal cAMP release occurred within one minute and this level remained steady, i.e. no further cAMP was released even if the cells were stimulated with isoproterenol for 30 minutes. The increase in cAMP release found when the non transfected cells were stimulated with isoproterenol (Fig. 60) was unexpected, and may have represented some contamination of these clones with transfected cells since B82 cells are not normally responsive to isoproterenol stimulation.

6.3 Documentation of a serum inhibitory effect on Wt-7 Cape Town cells

6.3.1 The effects of asthmatic serum on basal and isoproterenol stimulated cyclic AMP production

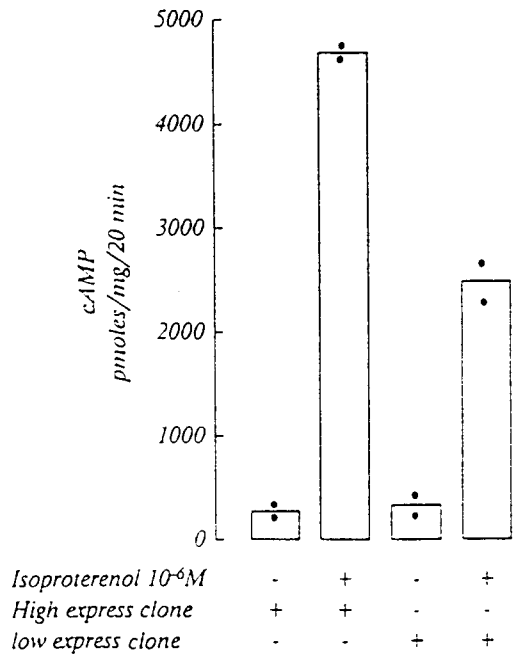


Figure 62. Isoproterenol stimulated cAMP release in "low" (480 fm/mg) and high (4800 fm/mg) human beta-2 adrenergic receptor expressing B82 cells. Bars indicate mean values of duplicate experiments (•).

The initial experiments were conducted to investigate whether fresh human serum would, in fact, influence human beta-2 receptor stimulated adenylate cyclase production in the Wt-7 Cape Town cells. For these experiments, blood was allowed to clot, serum immediately separated at 4°C and stored in aliquots at -70°C prior to the *in vitro* assays. Whole serum obtained from an asthmatic child (LJ) was used for the first exploratory experiments at 1:10 and 1:15 dilutions and (-)isoproterenol was used at 10^{-6}M. A dose response inhibition of isoproterenol stimulated cyclic AMP production occurred in the presence of the asthmatic serum (Fig. 63). There was no inhibitory or stimulatory effect on basal adenylate cyclase activity in the mouse cells with this patient (I did subsequently encounter sera which stimulated basal activity (e.g. Patient LB)). Inhibition of adenylate cyclase activity was observed whether transfected cells expressing high numbers of beta-2 receptors (Wt-7 Cape Town) or cells expressing lower (400 fmol/mg) numbers of receptors were used for the assays. This assay was repeated with a separate serum sample from the same patient using a different batch of Wt-7 Cape Town cells. A dose response shown in Fig. 64 confirms the inhibitory effect exerted by the serum on cAMP production.

Subsequent experiments were conducted to investigate whether different asthmatic sera varied in their ability to inhibit adenylate cyclase. For these experiments, my own serum (non-asthmatic), sera obtained from four asthmatic children (aged 8-14 years), two severe (steroid dependent) and two moderately severe asthmatics (not steroid dependent) were studied. Results of the functional assay and clinical profiles of the patients are shown in Fig. 65. Isoproterenol

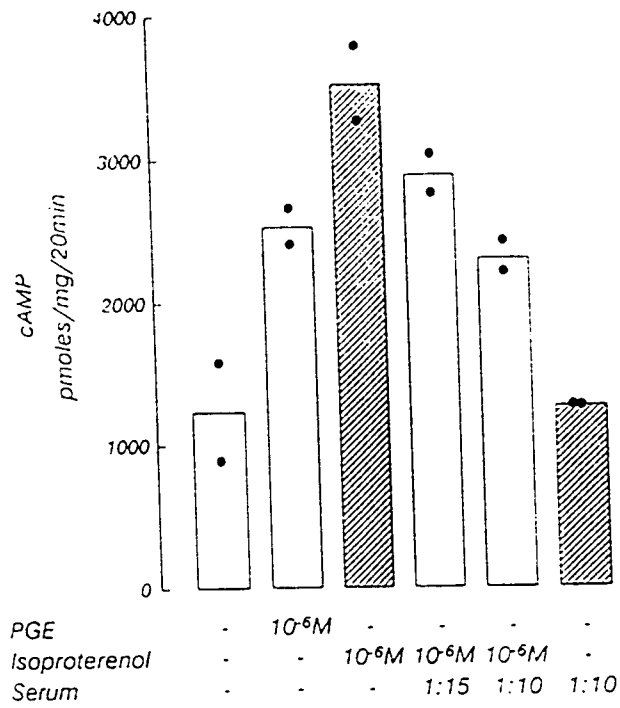


Figure 63. Basal, isoproterenol (10^{-6}M) and Prostaglandin E_1 stimulated cAMP production in Wt-7 Cape Town cells in the presence of asthmatic serum (1:15 dil. or 1:10 dil.). The effect of 1:10 dil. of serum on basal cAMP production is shown in the panel on the far right. Bars indicate mean values of duplicate experiments (•).

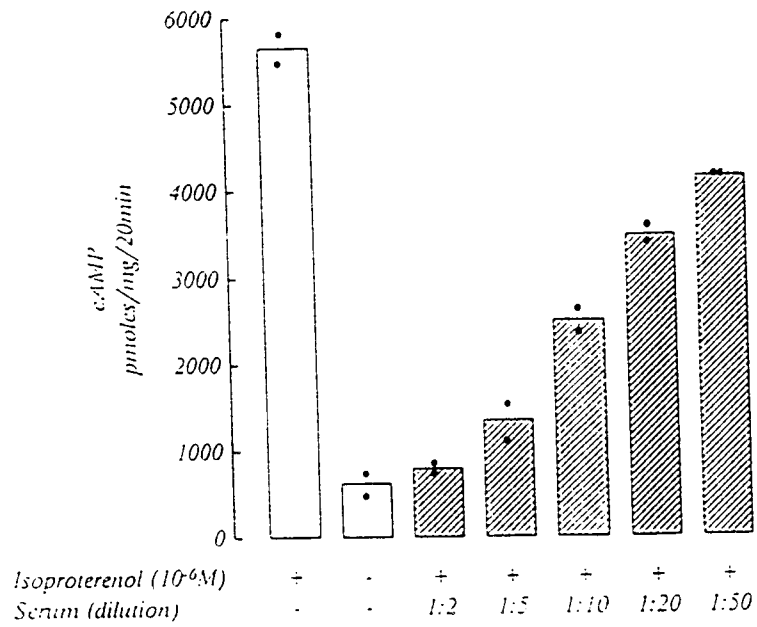
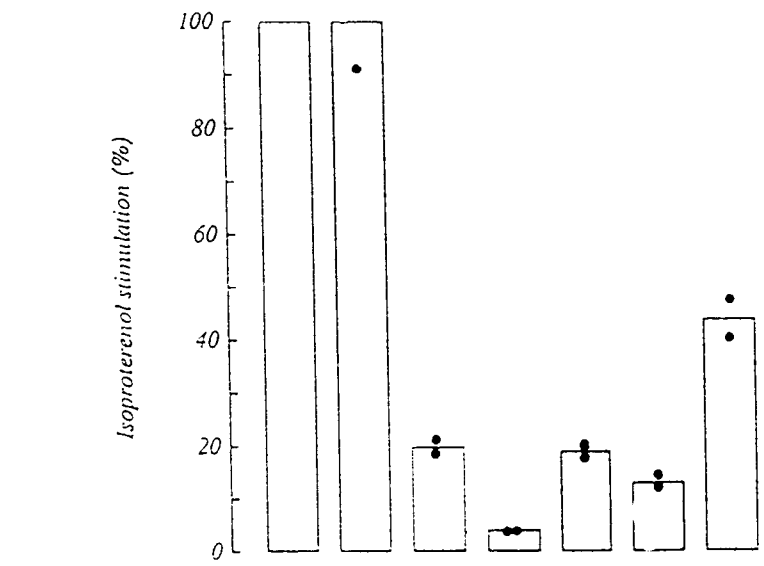


Figure 64. Dose responsive inhibitory effect of "asthmatic" serum on 10^{-6}M isoproterenol stimulated cAMP production in Wt-7 Cape Town cells. Bars indicate mean values of duplicate experiments (•).



Isoproterenol (10^{-6}M)	+	+	+	+	+	+	+
Serum (1:10 dil)	-	*NS*	*PA*	*A-RK*	*A-JV*	*A-HB*	*A-LG*
% ^{125}I-CYP inhibition	-	-	*46*	*73*	*37*	*46*	*40*

Age(yrs)	12	14	8	5
Severity grading	severe	moderate	moderate	severe
Drug therapy				
- theophylline	+	+	+	+
- salbutamol	+	+	+	+
- cromoglycate	+	+	+	+
- prednisone	+	-	-	+
- IgE (u/ml)	238	1838	296	415
RAST atopic status	+	+	+	+
Height (cm)	140	162	138	119
PEFR	350	420	320	240
Pred PEFR	315	425	269	207
FEV1	1.7	2.8	1.7	
Pred FEV1	2.1	2.8	1.7	-
FVC	2.3	3.9	2.0	-
Pred FVC	2.5	3.9	1.9	
FEV / FVC	73%	71%	70%	-

Figure 65. Inhibition of 10^{-6}M isoproterenol stimulation of cAMP production in Wt-7 Cape Town cells by 1:10 dilutions of human sera. NS = normal non-asthmatic serum (PCP). ; PA = pooled asthmatic serum; ARK, AJV, AHB, ALG = sera from moderate and severe asthmatic children. Bar values represent mean of duplicate assays (•).

stimulation was markedly inhibited by 1:10 dilutions of the fresh pooled asthmatic sera and by the individual sera, but was not inhibited by a 1:10 dilution of my own serum. Results are expressed as a percentage of the maximal cAMP produced in the presence of 10^{-6}M isoproterenol (without serum). The asthmatic sera were also tested in the [^{125}I]-CYP inhibition assay and results shown in Fig. 64 indicated that the patient (A-RK) whose serum was most inhibitory in the adenylate cyclase assay also displayed the greatest inhibition of [^{125}I]-CYP binding to the human beta-2 receptors.

6.3.2 Comparison of the effects of serum from well controlled and poorly controlled asthmatics on isoproterenol stimulated cyclic AMP production.

Since the degree of inhibition of cAMP production was found to be variable in 6.3.1, I conducted the following experiments to establish whether the degree of inhibition was in fact related to clinical status of the patients. I studied 10 asthmatics, five of whom were well controlled on their medication and five of whom had severe asthma and were difficult to control. Clinical profiles of the patients are shown in Table 16. Nine of the patients were on steroid therapy. For these experiments I noted that a higher level of expression of beta-2 adrenergic receptors was induced in the Wt-7 Cape Town cells (~8000fmol/mg) upon dexamethazone stimulation, than I had found initially, although these cells were derived from a frozen stock of Wt-7 Cape Town. (Subsequent subcloning of these cells reverted back to expression of approximately 4000fmol - 6000fmol/mg).

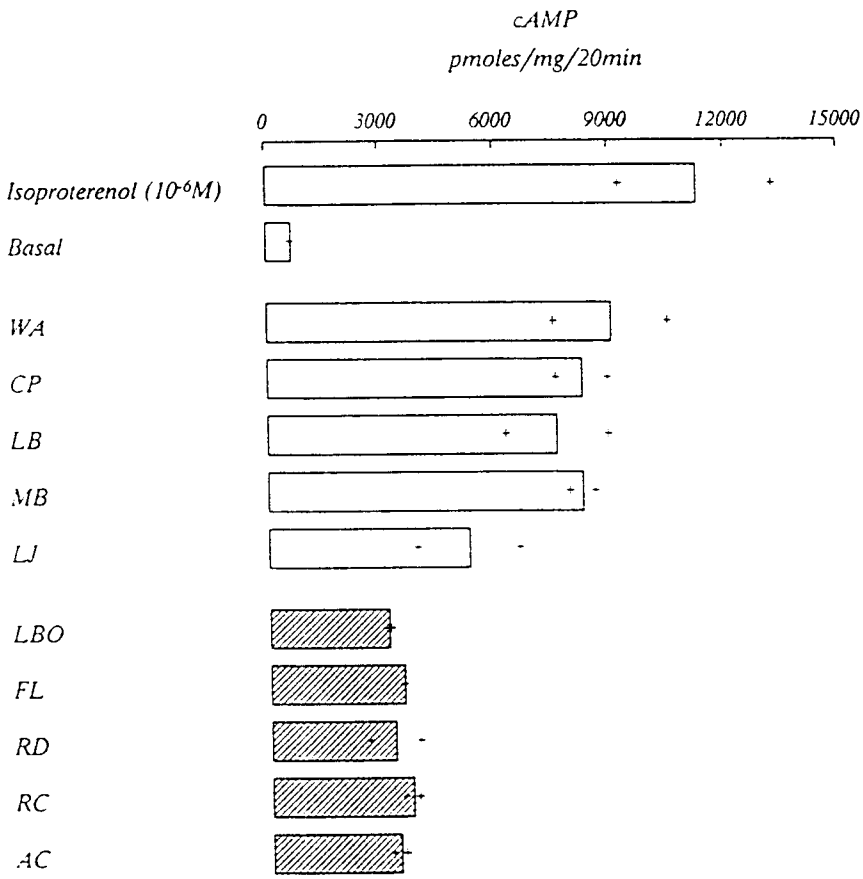


Figure 66. Inhibition of 10^{-6}M isoproterenol stimulated cAMP production by 1:10 dilutions of 10 asthmatic patients. Clinical details of each of these patients are detailed on Table 16. Bars indicate mean values of duplicate assays (+).

Table 16. Clinical status of patients in comparative assay.

	AL\C	RC	RD	FC\L	LBD	LJ	MB	LB	CP	WA
Age	13	12	8	13	10	12	14	9	9	8
Severity	very severe	severe deteri-orating	severe uncont-rolled	moderately severe	severe steroid dep.	severe steroid dep.	very well controlled mild	well controlled mod. severe	mild well controlled	moderate well controlled
Theophylline	+	+	+	+	+	+	-	-	-	+
Salbutamol	+	+	+	+	+	+	+	+	+	+
Cromoglycate	-	-	+	+	+	+	-	+	+	+
Steroids	Beclofort daily oral	Prednisone all days +	Prednisone	inhaled Becotide	inhaled Becotide	Prednisone daily	- - -	inhaled Becotide	inhaled Becotide	inhaled Becotide
IgE KU/L	35	-	3737	-	183	32	486	183	-	-
RAST/Atopic status	-	+	+		+	-	+	+	+	+
Height(CM)	125	145	119	147	132	125	161	132	136	119
*PEFR/PRED PEFR (or BEST)	230/280	120/359	195/207	370/267	340/274	340/365	460/481	340/274	295/294	220/207
FEV (or BEST) **FEV/PRED	1.5/1.7	0.7/2.3	-	1.9/2.2	2.0/1.8	1.6/1.95	-	2.0/1.82	-	-
FVC/PRED FVC	2.1/2.4	1.15/2.6	-	2.6/2.6	2.0/2.2	2.05/2.15	-	2.2/2.01	-	-
FEV/FVC	71%	60%	-	73%	90%	78%	-	90%	-	-

*Litres/min ** Litres/sec

Results given in Fig. 66 show that sera from both the well controlled asthmatic children and the poorly controlled severe asthmatic children were inhibitory but that with the severe asthmatics, the inhibition tended to be greater.

6.3.3 The effect of serum from non asthmatic children with other medical illnesses on isoproterenol stimulated cAMP production.

Since the initial inhibition experiments (6.3.1) showed no inhibition with control serum and suggested a greater degree of inhibition in the severe asthmatics, it was important to investigate whether or not these "inhibitory factors" were exclusively found in asthmatic serum. Sera from 5 normal (non-asthmatic) healthy children were tested for inhibitory effects on total cAMP production. Mean inhibition was 6.6% (individual values were 13%, 13%, 7%, 0%, 0%). Sera from ten hospitalised children were also studied. These children were admitted to the Children's Hospital with other medical conditions and none were asthmatic. Six sera were found to be inhibitory (> 50%) and the other four sera displayed low levels of inhibition (<20%). The inhibition of 10^{-6}M isoproterenol stimulation in the presence of 1:10 dilutions of sera from six of the ten children in Fig. 67 confirms that these "inhibitory serum factors" are not only present in the sera of children with atopic asthma. Sera which were found to be inhibitory included patients with acute glomerulonephritis, acute cellulitis of the foot, epilepsy and two relatively well children with diabetes who were on insulin treatment. These experiments clearly demonstrated that inhibitory factors in sera were not exclusive to asthmatics. I have directly compared the percentage of maximal

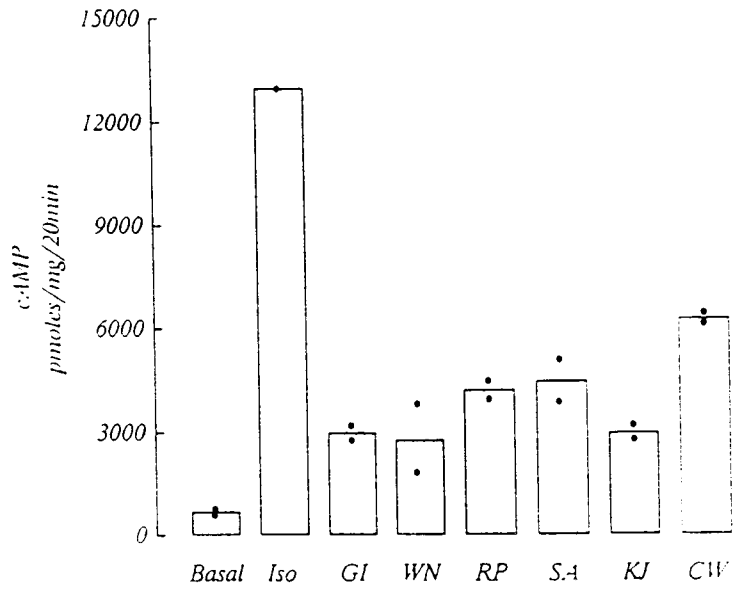


Figure 67. Basal and 10^{-6}M isoproterenol stimulated cAMP production in Wt-7 Cape Town cells. Inhibition of 10^{-6}M isoproterenol stimulated cAMP production was marked with 1:10 dilutions of sera from children with other medical conditions.

G1 - cellulitis; WN - acute glomerulonephritis; RP - epileptic; SA - epileptic; KJ - diabetic; CW allergic rhinitis.

Bars indicate mean values of duplicate assays.

stimulation of cAMP by 10^{-6}M isoproterenol in the presence of sera from 9 non-asthmatics with 11 asthmatics and found no significanat difference. Mean non-asthmatic: 5628pmol cAMP (SEM = 992); Mean asthmatic: 5219pmol cAMP (SEM = 744). p = 0.878 (Kruskal-Wallis test), p = 0.908 (Wilcoxin 2 - sample test).

6.3.4 Comparison of the effects of pre- and post-operative non-asthmatic sera on inhibition of cyclic AMP production.

In order to investigate whether the functional inhibitory activity in patient sera was generated by or amplified during an acute phase response, pre- and post-operative sera from six non-asthmatic adult patients in a gastrointestinal surgical ward were studied. The patients all underwent major surgery (cholecystectomy, oesophageal hernia repair, colostomy, or vasectomy and antrectomy).

Sera samples were taken on the day before major surgery and 24 hours after surgery and stored at -70°C. To confirm that the patients were undergoing an acute phase response, C-reactive protein (CRP) levels were measured (Beckman, laser nephelometer) on an aliquot of each sample. The effect of a 1:10 dilution of each patient's serum before or after surgery on 10^{-6}M isoproterenol stimulated adenylate cyclase in Wt-7 Cape Town cells was studied. Results given in Fig. 68 show that there was essentially no difference between the inhibitory capacity of pre-operative versus the post-operative sera.

It is also quite clear that individual patient sera again showed a spectrum of inherent inhibitory activity which did not vary

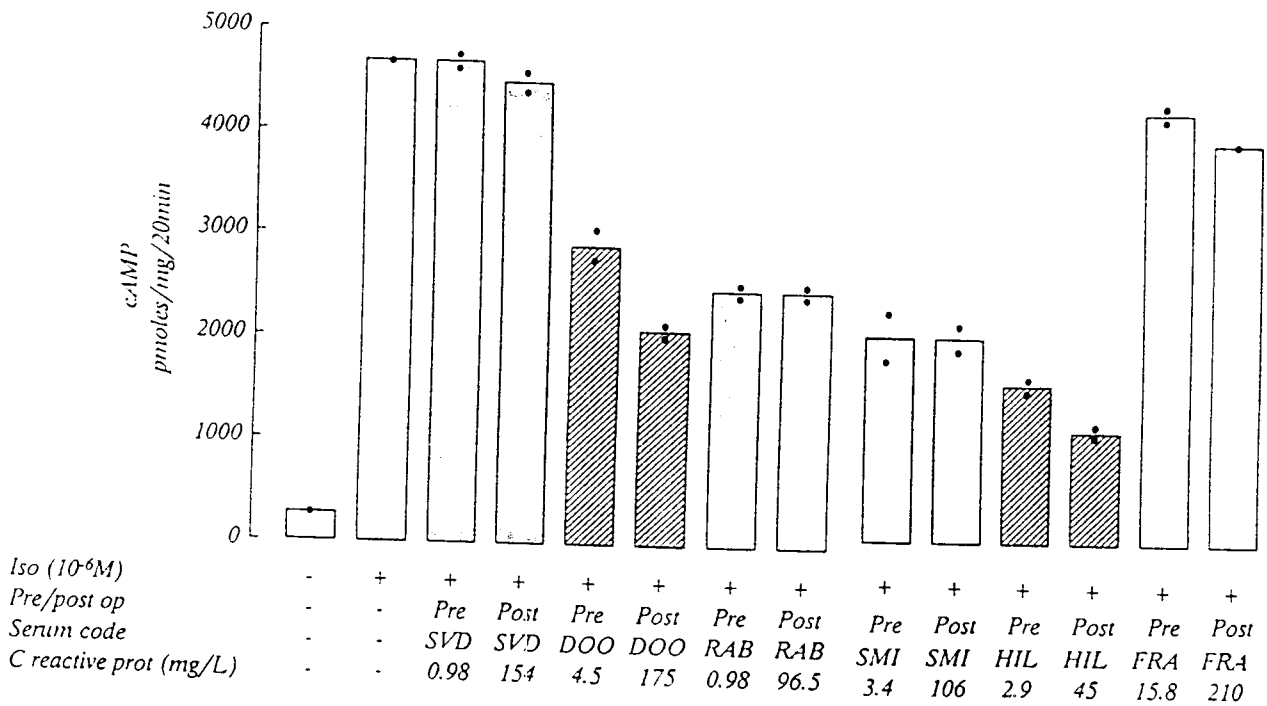


Figure 68. Comparison of the inhibitory effects of six pre- and post-operative sera on 10^{-6}M isoproterenol stimulated cAMP production. C reactive protein levels pre- and post-operatively are given in mg/L. Bars indicate mean values of duplicate assays (•).

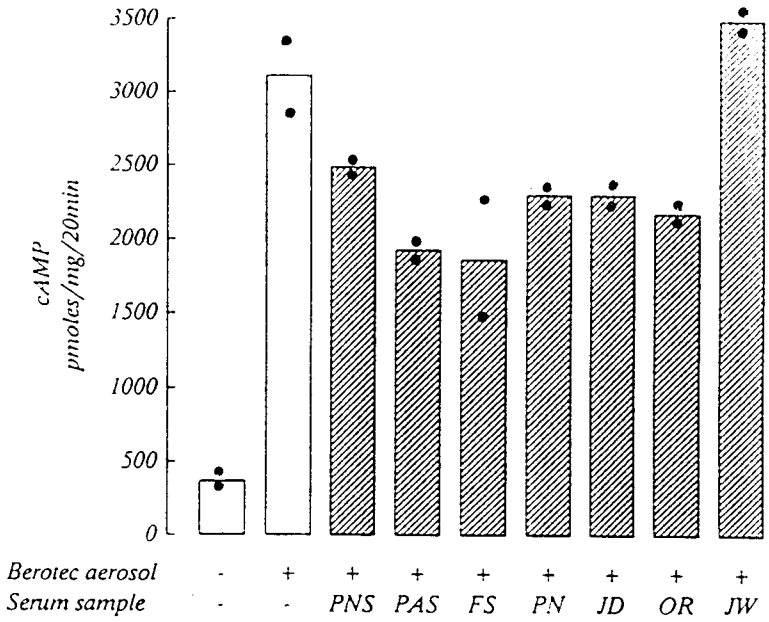
significantly over short periods of time (48 hours in this study). Sera code FRA and SVD were minimally inhibitory, while paired sera from HILL and SMI were markedly inhibitory pre- and post-operatively. Two patients had CRP levels less than 1 mg/L prior to surgery: one serum was inhibitory (Code RAB) while another (Code SVD) was not inhibitory. These data support a conclusion that the inhibitory activity of sera on adenylate cyclase activation is unrelated to the acute phase inflammatory response.

6.3.5 Comparison of the inhibition of isoproterenol and fenoterol (Berotec) stimulated cyclic AMP production by asthmatic sera

Since the binding of fenoterol to the human beta-2 receptor is more selective than that of (-)isoproterenol, I compared the difference between the inhibition mediated by asthmatic sera on isoproterenol and fenoterol mediated cAMP release from transfected Wt 7 Cape Town cells. Cells were stimulated by 10^{-6}M (-)isoproterenol as described (6.2.3), or a Berotec metered aerosol (Boehringer Ingelheim) was held 10 cm above the tissue culture dish and one puff (0.2mg Fenoterol hydrobromide) was delivered directly into the dish, after the cells had been incubated for 30 min with IBMX and a 1:10 dilution of patient's serum. Results of a comparative experiment shown in Fig. 69 demonstrate comparable degrees of inhibition by asthmatic sera for both agonists.

Of importance was that one of the asthmatic patient's sera (JW) consistently did not inhibit (-)isoproterenol or fenoterol stimulated cAMP release in this assay at this time point. This patient's serum

a



b

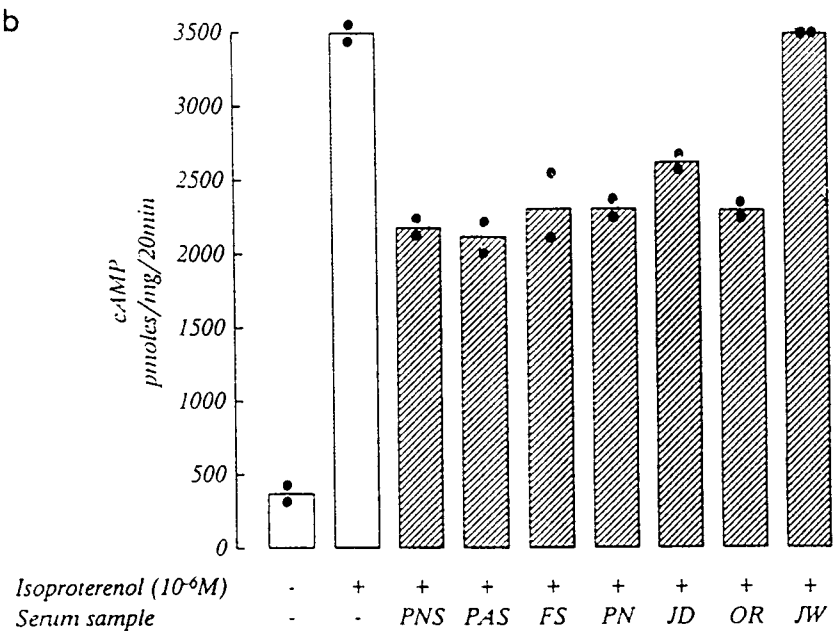


Figure 69. Similarity of the inhibitory effects of asthmatic sera on fenoterol (Berotec) (a) or isoproterenol (10^{-6}M) stimulated cAMP production (b). Bar values represent the mean of duplicate assays.

had been stored for one month at -20°C. Previously the sera from this patient was clearly inhibitory for (-)isoproterenol stimulated cAMP release. (Inhibition by his serum was also previously not removed by overnight dialysis of his fresh serum - see 6.4.4). These experiments suggested that the "inhibitory factor" may not be stable.

6.4 Identification of the nature of the serum inhibitory effect on Wt-7 Cape Town cells.

The "inhibitory factors" for cAMP production in serum were not present in all sera tested and appeared to be unstable on storage. To identify the origin and selectivity of the inhibitory effect it was important to locate which component of the adenylate cyclase coupled system was affected and to compare the effects of whole serum with that of plasma and serum fractions.

6.4.1 Comparison of the effect of serum on isoproterenol, PGE_1, sodium fluoride (G protein) and forskolin (adenylate cyclase) stimulated cAMP activity.

For these experiments fresh serum was used from an asthmatic patient (LB) whose serum previously consistently inhibited maximal isoproterenol stimulation by ±50%. Wt-7 Cape Town cells were stimulated with isoproterenol (10^{-6}M), PGE_1, forskolin (Sigma)(100μM) or sodium fluoride (Sigma)(1mM and 10mM). The differential effects on isoproterenol, forskolin or sodium fluoride are shown in Fig. 70 and these effects were completely reproducible and consistent in a subsequent separate experiment, when the concentration of

isoproterenol was varied between 10^{-10}M and 10^{-5}M and forskolin concentration between 1μM to 200μM. Serum inhibited 10^{-6}M isoproterenol, PGE_1, and forskolin stimulated cAMP release but, paradoxically, enhanced stimulation of cAMP release by sodium fluoride at both 1mM and 10mM concentrations. The percentage inhibition of maximal forskolin, and PGE_1, stimulated cAMP release, was identical to the inhibition of maximal isoproterenol stimulated cAMP release and suggested that the inhibitory effects of serum are not specific for the beta-2 receptor but that they are the result of a more generalised and non-specific membrane inhibitory effect.

6.4.2 The effect of pre-incubation and washing on the inhibitory effect of asthmatic serum on isoproterenol stimulated cyclic AMP activity.

Preincubation of the cells for 30 min with a 1:10 dilution of patient serum resulted in inhibition of 10^{-6}M isoproterenol stimulation, in the above assays. The effect of a 2 hour pre-incubation of Clone Wt-7 Cape Town cells with asthmatic serum (1/10 dilution, patient L-BD) was studied at two concentrations, 10^{-6}M and 10^{-8}M of (-)isoproterenol. Cells stimulated in the presence of 1/10 dilution of L-BD serum showed the expected decreased total cAMP production after 30 min pre-incubation with serum. However, when cells were pre-incubated with 1:10 dilution of serum for 2 hours, followed by two washes of phosphate buffered saline prior to stimulation in 10^{-6}M or 10^{-8}M isoproterenol, further reductions in the cAMP response were observed.. cAMP production in the presence of a 1:10 dilution of serum alone (in

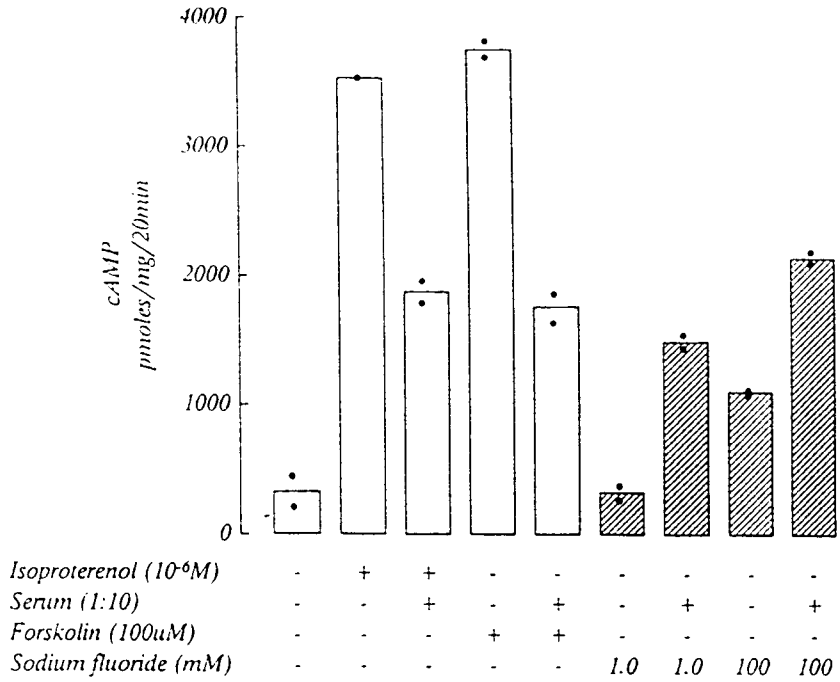


Figure 70. Inhibition of 10^{-6}M isoproterenol, 100μM forskolin and stimulation of 1mM or 100mM sodium fluoride stimulated cAMP production by 1:10 dilution of "asthmatic" serum. Bars indicate mean values of duplicate assays (•).

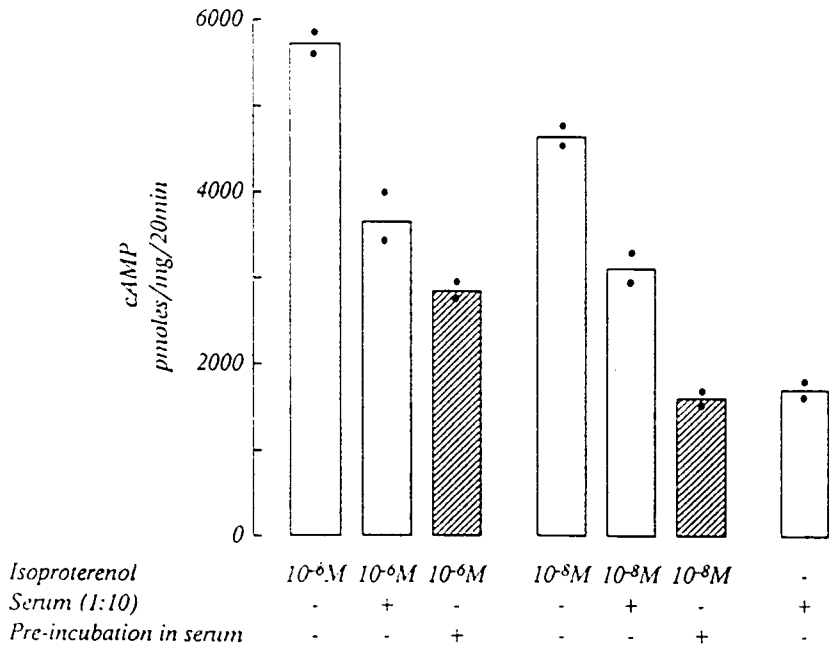


Figure 71. The effect of an extended pre-incubation (2 hours) on the inhibition of 10^{-6}M or 10^{-8}M isoproterenol stimulated cAMP production exerted by a 1:10 dilution of "asthmatic" serum (patient L-BD). Bars indicate mean values of duplicate assays (•).

the absence of isoproterenol) is shown in the bar panel on far right (Fig. 71).

The marked inhibition by serum which was not removed by washing the cells extensively with phosphate buffered serum suggested either an extremely high affinity of the "inhibitory factor" for the cells or that serum mediated a toxic effect on the cells which was irreversible.

6.4.3. The effect of clotting on the inhibition of (-)isoproterenol stimulated cyclic AMP activity by human sera/plasma

In order to investigate the generation of the "serum inhibitory factors" it was important to test whether or not these factors were also present in plasma. For these experiments fresh venous blood was taken from a child with asthma and either allowed to clot for 10 min (and serum separated immediately), heparinised, or was treated with EDTA to inhibit clotting, and fresh plasma separated immediately and held at 4°C for 30 minutes prior to conducting the assay. Results illustrated in Fig. 72 show that while a 1:10 dilution of the serum was inhibitory, heparin, heparinised plasma, or EDTA plasma slightly enhanced 10^{-6}M (-)isoproterenol stimulated cAMP release. The absence of an inhibitory effect with plasma was confirmed with studies of other sera in subsequent experiments.

6.4.4 The effect of dialysis of serum on inhibition of isoproterenol stimulated adenylate cyclase

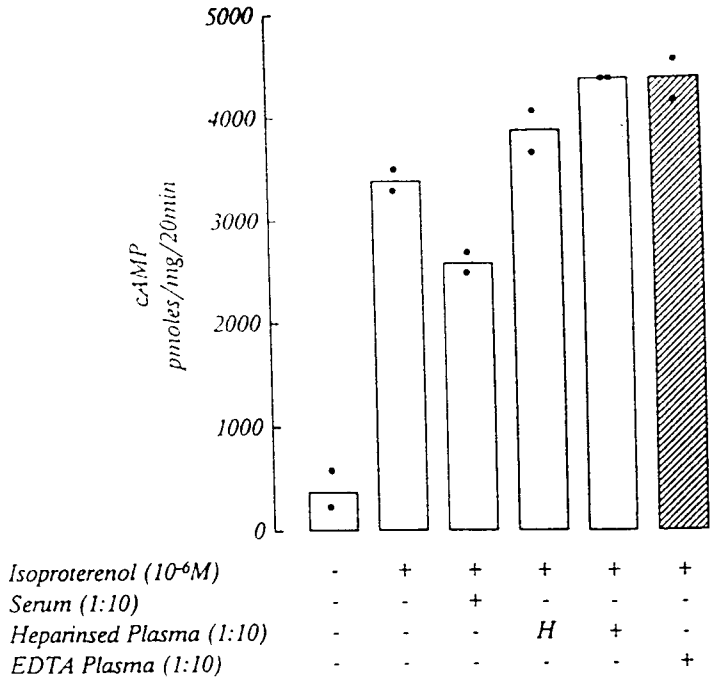


Figure 72. Comparison of the effects of serum (inhibitory) and plasma (heparinised ▩, or EDTA ▨) on 10^{-6}M isoproterenol stimulated cAMP production. The effect of heparin alone is designated "H". Bars indicate mean values of duplicate assays (•).

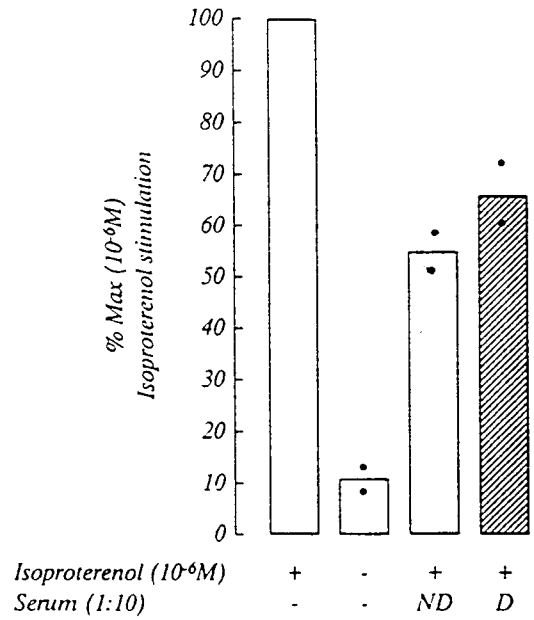


Figure 73. The effect of dialysis (D) ▨ on the inhibition of 1:10 dilution of serum (ND) ▩ on 10^{-6}M isoproterenol stimulated cAMP production. Bars indicate mean values of duplicate assays (•).

Fresh serum was dialysed against 20mM sodium phosphate buffer overnight using Spectropor(R) membrane tubing (Spectrum Med. Ind. Inc.) molecular weight cut-off 12000-14000 daltons. As found with antagonist [^{125}I]-CYP inhibition (3.2.2(i)), the functional inhibitory factor(s) were not removed by dialysis (Fig. 73, patient JW) indicating that these factors have a molecular weight of greater than 12000 daltons.

6.4.5 The effect of fractions of asthmatic serum on isoproterenol stimulated cyclic AMP activity.

Three fractions of fresh serum from two severe asthmatics whose sera were found to be consistently inhibitory were studied. Sera were precipitated in 33% ammonium sulphate (Fraction I) followed by a 66% ammonium sulphate precipitate of the supernatant (of the 33% ammonium sulphate precipitate) (Fraction II). Fraction III (Sup) was the supernatant of the second (66%) precipitation. All fractions were dialysed overnight in PBS to remove the salt (using Spectrapor (R) membrane tubing, 12000 - 14000MW cut-off).

These three fractions were then reconstituted in assay buffer to the original serum volume and a 1:10 dilution was used in the adenylate cyclase assay. Inhibitory effects on total cAMP production were studied at 10^{-6}M concentrations of isoproterenol. No consistent inhibition of cAMP production was mediated by any of the serum factors studied, indicating that the inhibitory factor(s) were not mediated by the immunoglobulin fractions alone and that the "inhibitory factors" were unstable.

6.4.6 Thermal stability of the inhibitory factors in serum.

When performing reproducibility studies on stored samples, I noted that the inhibitory effects observed were greater when fresh serum or serum stored at -70°C was used in the assays, but that when serum was stored at -20°C for a month or longer, the inhibitory activity declined significantly. Inhibitory effects were markedly reduced when serum was stored at 4°C for 72 hours and totally inactivated if the serum was heated to 56°C for 30 min (Fig. 74).

6.4.7 Western blots of Wt-7 Cape Town cells incubated with human sera

Since the stability studies suggested that the inhibitory effects on cAMP production could be complement mediated, cell membranes were prepared from the Wt-7 Cape Town cells and Western blotted on to nitrocellulose membranes to investigate whether there were antibodies in the human sera which recognised mouse proteins and which could be involved in activation of the classical pathway of complement leading to cell damage of the Wt-7 Cape Town cells.

Western blots of Wt-7 Cape Town cell membranes were incubated with 1:50 dilutions of patient sera for 90 min, washed 3 times followed by alkaline phosphatase-conjugated goat anti-human Ig (G + A + M) (Zymed) 1:1000 dilution and addition of BCIP (5 Bromo 4 chloro indoxyl phosphate) substrate (Fig. 75). In no instance was there a clear recognition of human beta-2 adrenergic receptors (each lane had 1 picomole of beta-2 receptors). Certain sera showed no recognition of

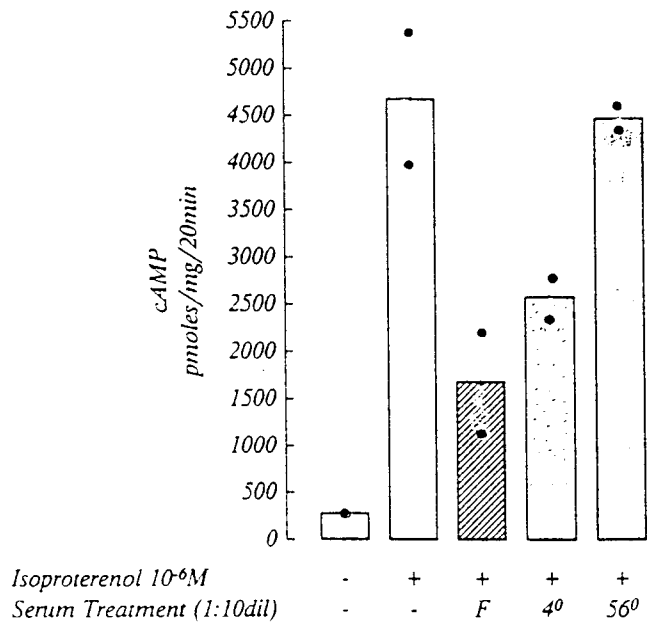


Figure 74. The effect of method of storage of serum on the inhibitory effects of serum on 10^{-6}M isoproterenol stimulated cAMP production F ▨ (Frozen at -70°C); 4°C (stored at 4°C for 72 hours ⊡); 56°C for 30 minutes ■. Bars indicate mean values of duplicate experiments.

any mouse proteins in the blot (lane 1) whereas other sera from asthmatics recognised several distinct mouse proteins (lane 2, 3 and 4). In other Western blots (Fig. 75(a)) using overnight incubation with first antibody human sera recognized many mouse antigens.

Thus it was possible that certain sera had heterophile (anti-mouse) antibodies which, if they activated complement, could lyse the mouse L cells and result in damage to cell membrane function. This would account for the functional serum inhibitory effects I had observed on the Wt-7 Cape Town cells. It was thus important to study the effects of human serum on Wt-7 Cape Town cell viability.

6.4.8 Effects of human serum on viability of Wt-7 Cape Town cells and the effect of heat inactivation on the serum inhibitory factors

Since the experimental evidence thus far suggested that the "inhibitory effects" of serum could be due to complement mediated lysis of mouse cells by heterophile anti-mouse antibodies, I investigated the effects of specific individual sera which I had previously identified to be inhibitory, or non-inhibitory in the cAMP experiments, on cell viability over 2 hours using both a human melanoma cell line (UCT-Mel 2), and the mouse cells (Wt-7 Cape Town). The design and results of this experiment are summarised in Table 17. It was quite clear under direct microscopy that within 30 min of applying fresh serum (1:10 dilution) to the mouse cells they rounded up, became less refractile, acquired granules, became non-adherent and were membrane damaged (failed to exclude Trypan blue). These changes were not observed when serum was heated to 56°C for 30 min or when a

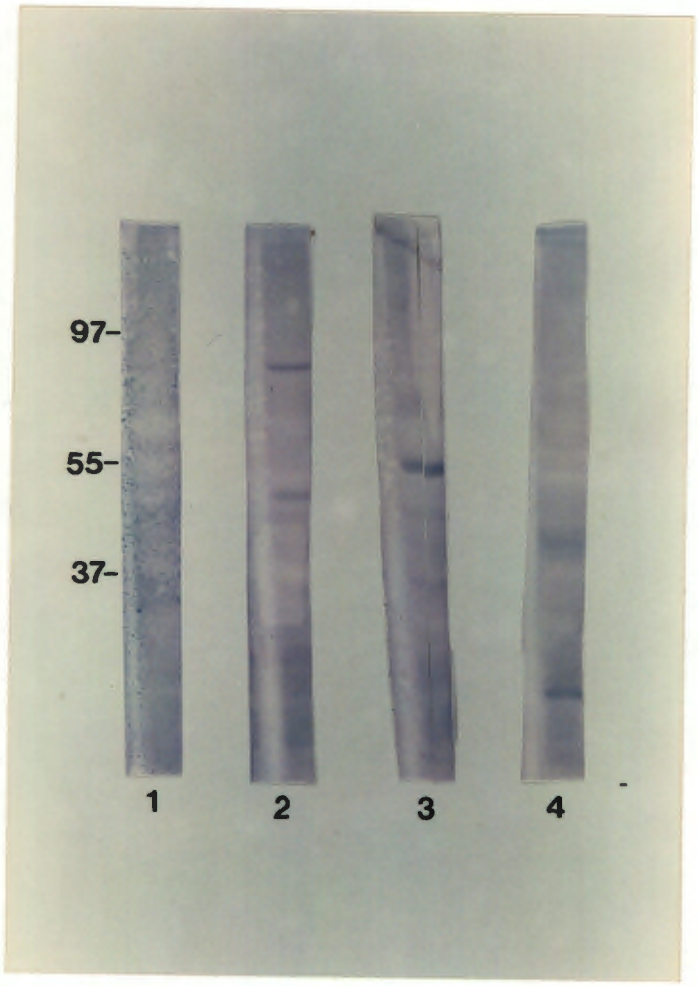


Figure 75. Western blots of 11% PAGE Wt-7 Cape Town cell membranes incubated for 1.5 hours with 1:50 dilution of sera from 4 asthmatic patients. Lane 1: no bands detected; late 2: 2 bands detected; Lanes 3 and 4: single different bands detected.

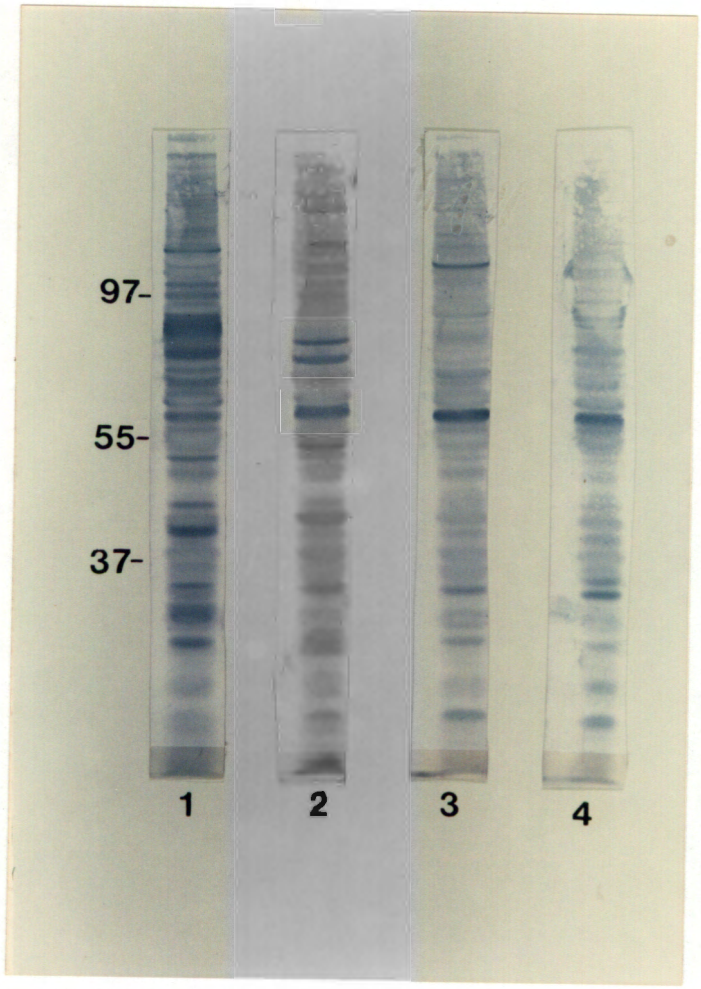


Figure 75a Western blot of 11% PAGE of Wt-7 Cape Town cell membranes incubated overnight with 1:50 dilutions of sera from asthmatic patients showing binding to many proteins in the mouse membrane preparation.

TABLE 17

Serum/Plasma used	Effect of serum (1:10)	Effect of 1:10 dilution of serum on cell morphology at 90 min	
		Wt-7 Cape Town (mouse)	UCT-Mel 2 Melanoma (human)
Fresh asthmatic serum(LB)	inhibitory	cell death	normal
Fresh non-asthmatic serum	non-inhibitory	normal	normal
Fresh asthmatic plasma (LB)	non-inhibitory	normal	normal
Fresh non-asthmatic serum (Hill)	inhibitory	cell death	normal
Heat inactivated LB serum	non-inhibitory	normal	normal
Heat inactivated Hill sera	non-inhibitory	normal	normal
Asthmatic sera serum JW	non-inhibitory	partial cell death	normal
Heated asthmatic	non-inhibitory	normal	normal

1:10 dilution fresh plasma was used instead of serum. Fresh serum did not lyse the human melanoma cells, supporting the conclusion that one likely explanation for the inhibitory effects observed by certain human sera in the mouse system was complement mediated lysis of the mouse cells activated by heterophile antibodies.

I have found consistently that heat inactivation reduces the inhibitory effects of human sera on adenylate cyclase activation and cAMP production, but that there is residual inhibitory activity even in the heat inactivated serum, suggesting that there may be other (non complement mediated) mechanisms for the inhibitory effects in human sera (Fig. 76).

In a comparison of the % maximal stimulation for 10^{-6}M isoproterenol stimulated cAMP production using fresh or stored sera (-20°C) from 10 patients, the mean % stimulation for fresh sera was 63.1%, SEM = 8.1 and for stored sera 92.5%, SEM = 1.7. This difference was highly significant $p = < 0.01$ (Student's t).

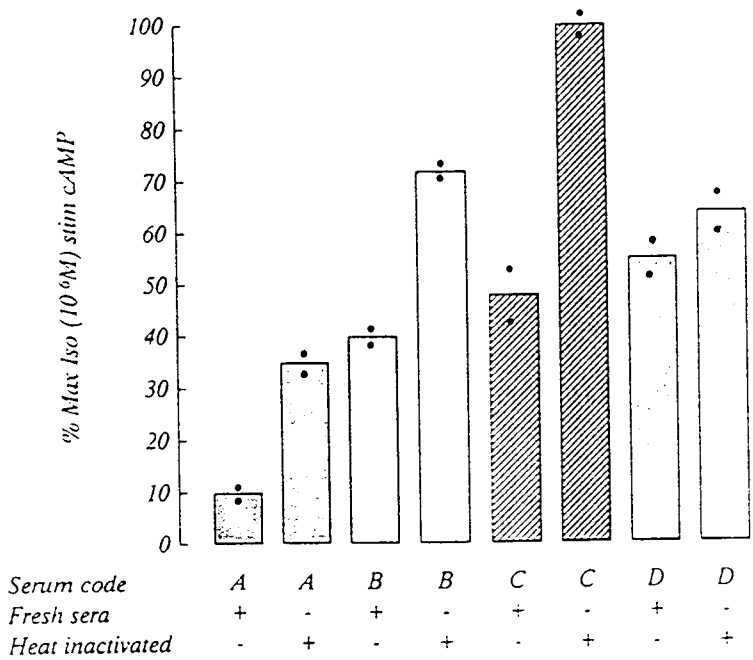


Figure 76. Partial removal of the inhibitory activity of 4 asthmatic sera on 10^{-6}M isoproterenol stimulated cAMP production by heat inactivation (56°C for 30 min). Bar values represent mean of duplicate assays (•).

The experiments on cell viability after incubation with human serum thus showed that inhibition of cAMP activity by fresh sera described in 6.3.1 - 6.3.5 can not be attributed solely to a specific effect on the adenylate cyclase system. A heat labile "factor" is present in serum, but not in plasma, which damaged cell membranes and resulted in a loss of cAMP activity. These observations are consistent with complement mediated cell membrane damage via heterophile antibodies, but may also be caused by other serum components.

The Wt-7 Cape Town cells are thus not suitable for investigating the effects of heat labile components in human sera on adenylate cyclase activation in view of the presence of heterophile antibodies to mouse antigens in many of the human sera. It is likely that similar difficulties may be encountered with other non-human cellular systems.

This system is however suitable for the study of the direct effect of purified specific components of human sera, or activated cells, on the beta-2 receptor coupled adenylate cyclase system. I have therefore proceeded to study the effects of purified IgG, monoclonal IgM, rabbit polyclonal anti beta-2 receptor peptide antibody, activated neutrophils, activated lymphocytes and activated platelets on the adenylate cyclase system in Wt-7 Cape Town cells in section 6.5, and have conducted further ligand binding studies on membrane preparations of the Wt-7 Cape Town cells, as a follow-up to the experiments described in Chapter 3.

6.5.1 The effect of whole human IgG and $F(ab)_2$ IgG fragments on isoproterenol stimulated cyclic AMP activity.

The effect of purified immunoglobulin G (IgG) and $F(ab)_2$ fragments of IgG on isoproterenol-stimulated adenylate cyclase activation in Wt-7 Cape Town cells was investigated. Purified IgG fractions from a pool of asthmatic patients whose sera were shown to be inhibitory for adenylate cyclase activation, and the $F(ab)_2$ fragments prepared from the purified IgG, were used.

DEAE purified IgG was digested overnight with pepsin (Sigma), at pH 4.5, and after centrifuging, the pH of the supernatant was adjusted to 7.4 (to inactivate the pepsin), dialysed overnight with phosphate buffered saline pH 7.4, and applied to a 40cm Sephadex G100 column. Two peaks were separated: the first contained the $F(ab)_2$ fragments (Sterin Borda et al. 1988). Purity of the IgG and $F(ab)_2$ fragments was checked on PAGE.

Samples from five asthmatic subjects containing equivalent IgG and $F(ab)_2$ fragment protein concentrations to the IgG concentration in a 1:10 dilution of serum measured by Nephelometry (Beckman Immunochemistry Analyser) were compared with inhibition of isoproterenol stimulated cAMP release obtained with a 1:10 dilution of fresh serum. No significant inhibition and cAMP activity was mediated by either whole immunoglobulin G or $F(ab)_2$ fragments. The

finding contrasts sharply with effects of purified immunoglobulin G fractions in the [^{125}I]-CYP inhibition assay (3.3).

These data support a conclusion that factors in serum identified in Chapter 3 which inhibit antagonist binding ([^{125}I]-CYP) to membrane bound beta-2 adrenergic receptors (and which may include the IgG fraction) are distinct from those which inhibit the functional beta-2 adrenergic receptor activity in live Wt-7 Cape Town cells.

6.5.2 The effect of rabbit polyclonal and mouse monoclonal anti-beta receptor peptide BR-20-11 antibodies on basal and maximal adenylate cyclase activation.

Although immunogloblins from human sera showed no inhibitory effects on adenylate cyclase, it was important to investigate whether antibodies specifically raised to a peptide derived from the beta-2 receptor had any functional inhibitory, or stimulatory activity.

The polyclonal antibodies I had raised in two rabbits (700 and 701) to BR-20-11 peptide (5.7) and the monoclonal antibody B4A8-PWL (ascites fluid)(5.6) were tested at 1:10 dilutions for their effects on basal adenylate cyclase activity and 10^{-6}M isoproterenol stimulated cAMP release in Wt-7 Cape Town. There was no inhibition or enhancement of maximal isoproterenol stimulated cAMP production with rabbit serum (1:10 dilution) or with monoclonal antibody purified IgM (0.2g/L). A degree of stimulation above basal was obtained with the monoclonal at 1:10 dilution and with the rabbit polyclonal (rabbit 701) antibody

(1:10 dilution). To confirm the specificity of the stimulatory activity, the rabbit was boosted three times with the peptide (BR-20-11), and the effect of 1:10 dilution of the hyperimmune rabbit serum was compared with the original serum. Results given in Fig. 77 show that the stimulatory activity increased from 85% above basal cAMP level, to 162% above basal levels.

The rabbit anti-peptide antibodies were also tested for their ability to inhibit [^{125}I]-CYP binding. Both rabbit sera were inhibitory, but that rabbit 701 was markedly inhibitory for [^{125}I]-CYP binding (86% inhibition at 1:10 dilution)(Fig. 78).

By contrast, control non-immunised rabbit sera and 1:10 dilution of rabbit anti-peptide sera had minimal effects on 10^{-6}M isoproterenol stimulation of Wt-7 Cape Town cells (less than 5% inhibition). Purified B4A8 monoclonal IgM, at a concentration of 0.2g/L, similarly had no inhibitory effect on maximal isoproterenol stimulation. (Monoclonal IgM was purified using a Sephadex G200 column and fractions containing IgM were identified using PAGE and dot blotting, using a goat anti-mouse IgM antibody).

These results demonstrate that the polyclonal antibodies may have fairly marked effects on basal adenylate cyclase activation. They also demonstrate that certain sera may have significant effects on high affinity antagonist binding (86% inhibition) but exert minimal effects on maximal functional activity and at the same time demonstrate intrinsic cAMP stimulatory activity.

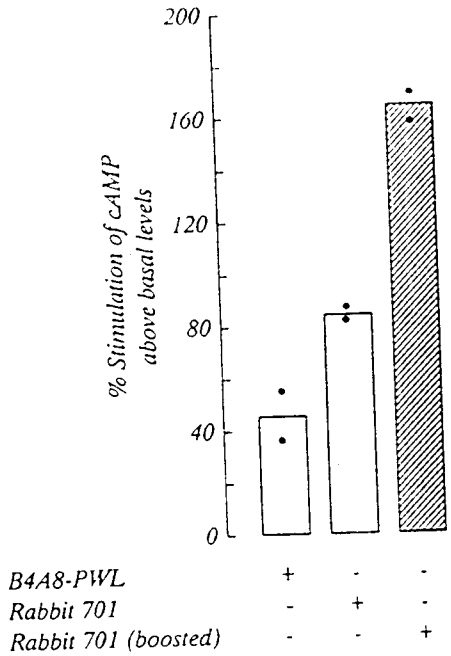


Figure 77. The effect of monoclonal B4A8-PWL (1:10 dil ascites)(Bar 1) rabbit 701 antibody (1:10 dil.)(Bar 2)(and boosted rabbit 701 antibody (1:10 dil.)(Bar 3) on basal adenylate cyclase activity (Values expressed as % stimulation above basal levels represent the mean of duplicate assays (•)).

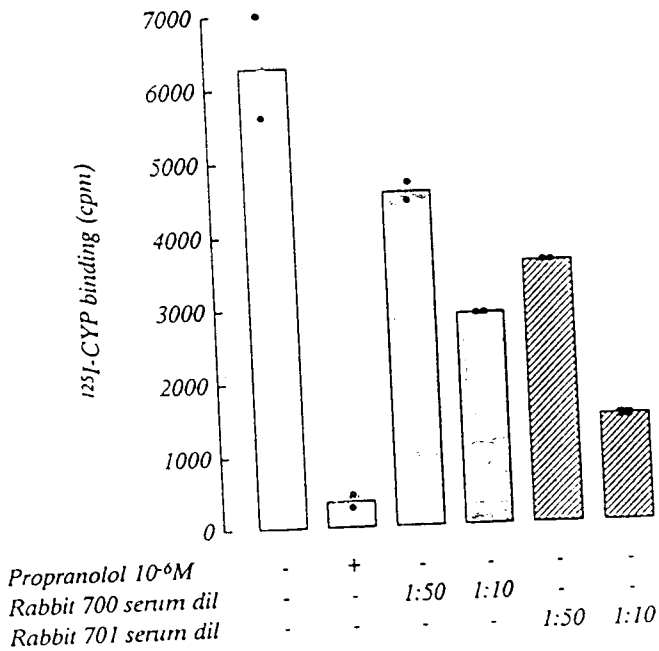


Figure 78. Inhibition of [^{125}I]-CYP binding to Wt-7 - Cape Town membranes by 1:50 and 1:10 dilutions of rabbit 700 (▨) or rabbit 701 (▩) serum. Bar values represent the mean of

I have also observed that while the majority of human sera studied had no effect on basal cAMP release, one patient (LB) serum consistently stimulated basal cAMP release in the absence of isoproterenol (20% of maximal isoproterenol stimulation).

6.5.3 The effect of products of activated normal neutrophils and Con A stimulated normal lymphocytes on isoproterenol stimulated cAMP release.

In an attempt to identify other possible cellular sources of adenylate cyclase inhibitory activity, the effect of supernatants from activated neutrophils and lymphocytes were also investigated.

Normal lymphocytes were cultured in RPMI for three days in 20μg/ml Concanavalin A at 10^6 cells/ml in 10% foetal calf serum. After three days, the cultures were centrifuged to remove cells and methyl α-D-manopyranoside (MMP) was added to a final concentration of 10mM to inactivate the Con A. The medium was then filtered through a 0.45μM Millipore filter prior to stimulating the Wt-7 Cape Town cells with 10^{-6}M isoproterenol either in neat supernatant, or a 1:2 dilution of supernatant.

Normal neutrophils, separated on Ficoll hypaque were suspended in PBS at a concentration of 10^6 neutrophils/ml and stimulated with 10ng/ml or 100ng/ml phorbol myristate acid (PMA). Cells were separated by centrifugation and the effect of the supernatants on 10^{-6}M isoproterenol stimulation of Wt-7 Cape Town cells examined.

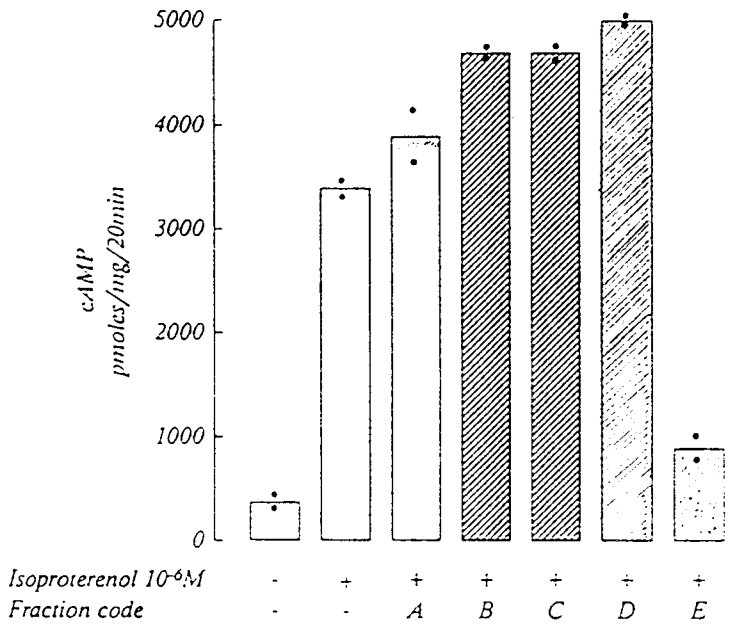


Figure 79. The effect of products of PMA activated neutrophils, RPMI, and lymphocyte conditioned media on 10^{-6}M isoproterenol stimulated cAMP production - A - RPMI; B - lymphocyte conditioned media (neat); C - Con A stimulated lymphocyte conditioned media (neat); D - Con A stimulated lymphocyte conditioned media (1:2); E - PMA stimulated (100 ng/ml) neutrophil products. (Bars represent mean values of duplicate experiments (.)).

Results given in Fig. 79 show that RPMI mildly enhanced isoproterenol stimulation, but that neat lymphocyte conditioned media, with or without Con A, significantly enhanced isoproterenol stimulation by 43% and diluted lymphocyte (1:2) conditioned media enhanced stimulation by 53%.

The neat products of 100 ng/ml PMA stimulated neutrophils (which release lysosomal granules)(but not the 1:5 dilution) were markedly inhibitory for 10^{-6}M isoproterenol mediated adenylate cyclase activity (% stimulation of maximum = 17%). Supernatants from neutrophils activated with 10ng/ml PMA (which do not release lysosomal products) were not inhibitory. (PMA is unstable under the conditions of the experiment and the results cannot be attributed to the effect of PMA alone).

These data do suggest that neutrophil products are a possible candidate source of inhibitory factors for adenylate cyclase activation, but that lymphocytes do not release inhibitory factors (under normal culture conditions) but may in fact stimulate cAMP production (possibly through the release of prostaglandins or other molecules).

The role of neutrophil products in mediating beta-2 receptor inhibition needs to be explored in further studies. I have, unfortunately, due to financial and time constraints been unable to extend these studies with neutrophils, but have in these studies identified a promising area for further research. The inhibitory effects of inflammatory cell mediators have recently been reported in

the studies by Raaijmakers (1989) who demonstrated that arachidonic acid metabolites PAF, LTB_4 and LTC_4 inhibit beta receptor ligand binding to pulmonary membranes and also inhibit isoproterenol and Gpp(NH)p activation of lymphocytes adenylate cyclase.

6.5.4. Effects of products of ADP activated platelets on (-) isoproterenol stimulated cAMP release.

In view of the marked inhibitory effects of activated neutrophils, I have also examined the possibility that inhibitory factors may arise from platelets which are activated during the clotting process or during cellular inflammation by IgE or platelet activating factor (PAF).

For these experiments, platelets at a concentration of $1x10^9$ platelets/ml (in donor serum) were obtained freshly from the Western Province Blood Transfusion Service, with the assistance of Mr. Alan Kirby, one hour before the experiment was conducted, and maintained at 37°C.

Platelets were activated (as monitored by light scatter), for 15 min with 5μM ADP (adenosine diphosphate) at 37°C, or inactivated at 47°C for 10 minutes, prior to stimulating with 5μM ADP at 37°C. The effect of a 1:10 dilution of platelet products released into serum from activated, inactivated platelets was compared with the effect of a 1:10 dilution of platelet poor serum or 10^{-6}M isoproterenol stimulation of WT-7 Cape Town cells.

Results of these experiments show that minimal and identical inhibition (<15%) was mediated by serum in which platelets were activated or inactivated, and that this inhibition was of the same order as the inhibition mediated by a 1:10 dilution of the platelet poor serum (20%). These data showed that inhibitory factors for adenylate cyclase are not derived from activated platelets, at least under the conditions of the study.

6.5.5 Summary of the inhibitory and stimulatory effects of cellular components and serum on isoproterenol stimulated cAMP production in Wt-7 Cape Town cells.

1. Certain fresh human sera were found to dramatically inhibit isoproterenol stimulated cAMP production in Wt-7-Cape Town cells.

2. Inhibitory activity of serum was stable when stored at -70°C, relatively unstable at 4°C and totally inactivated when serum was heated at 56°C for 30 min, consistent with complement damage to the cells.

3. Viability studies showed that inhibitory human sera (at 1:10 dilution) produced morphological cell changes, visible under light microscopy when applied to mouse cells (L cells) but not to the human cells (Melanoma cells) studied.

4. Inhibitory activity on mouse cells was not present in all sera but was found in asthmatic sera and non-asthmatic hospitalised children with other medical conditions. Healthy normal and hospitalised sick children were identified whose sera displayed no inhibitory activity. (Inhibitory activity tended to be greater in the older patients.)

5. Inhibitory activity of serum did not vary over the short term (48 hrs); was highly reproducible in a given patient, and was not amplified during an acute phase inflammatory response (as measured by C reactive protein levels).

6. Cyclic AMP inhibitory activity was not present in plasma, (EDTA or heparinised), not generated by activated platelets, Con A activated lymphocytes, RPMI culture medium, normal rabbit serum, DEAE purified IgG or $F(ab)_2$ fragments, or 33% and 66% ammonium sulphate precipitates of serum.

7. Significant inhibition of cAMP activity was mediated by the products released from PMA activated neutrophils.

8. Although PGE_1, isoproterenol and forskolin stimulated adenylate cyclase activity in Wt-7 Cape Town cells was inhibited by fresh serum, sodium fluoride stimulated cAMP release was paradoxically enhanced in the presence of fresh serum.

9. Rabbit polyclonal antibodies raised to a beta-2 receptor peptide (BR-20-II) stimulated adenylate cyclase (raising cyclic AMP levels well above basal levels) but did not inhibit isoproterenol stimulated adenylate cyclase activity.

6.6 Follow up studies of serum inhibition of [^{125}I]-CYP binding to cloned human beta-2 receptors in membranes of Wt 7 Cape Town cells.

6.6.1 Inhibition of [^{125}I]-CYP binding to cloned human beta-2 receptors by sera from normal children, normal adults and asthmatic children

The studies conducted in Chapter 3 on the serum inhibitory effects on [^{125}I]-CYP binding raised certain specific questions (3.6). Using the stable source of human beta-2 receptors from the Wt-7 Cape Town clone, I have re-examined the reproducibility and significance of the antagonist ligand inhibitory effects of serum from 34 individuals including those patients whose sera previously exerted greater that 50% inhibition in Table 8.

The inhibitory effects observed with the cloned human beta-2 adrenergic receptors are similar to those found with the guinea pig lung beta adrenergic receptors:

Human sera inhibited [^{125}I]-CYP binding to cloned human beta-2 receptors in a dose responsive fashion. [^{125}I]-CYP binding to the

Wt-7 Cape Town human cells is of high affinity (Kd=20pM). Maximal binding was achieved by 30 minutes and was stable for at least 2 hours. Inhibition of ligand binding by serum was rapid and was observed within one minute (no pre-incubation of membranes was necessary). Inhibition mediated by serum or plasma (EDTA or heparinised) was of the same order. Inhibition of [^{125}I]-CYP binding was not removed by prior dialysis of the serum, after 10-fold dilution of the serum, overnight. Inhibition of [^{125}I]-CYP binding was not altered by the presence or absence of the protease inhibitor PMSF in the binding assay, and serum which spun through Sephadex G50 did not bind [^{125}I]-CYP directly.

Studies of the antagonist inhibitory effects of human asthmatic sera were extended to an investigation of the effect of heat inactivation of the serum (30min at 56°C) and passing the serum through a Sephadex G50 column, on [^{125}I]-CYP binding to cloned human beta-2 receptors.

Results given in Fig. 80 show that heat inactivation, dialysis and passing sera through a G50 column did not remove the inhibitory effect of pooled human asthmatic serum on [^{125}I]-CYP binding to human beta-2 receptors.

Wt-7 Cape Town membranes were incubated with a 1:50 dilution of serum for 30 min at 30°C after which the membranes were spun down, resuspended and washed in fresh buffer, before incubating with [^{125}I]-CYP. Inhibition of [^{125}I]-CYP binding by serum was only

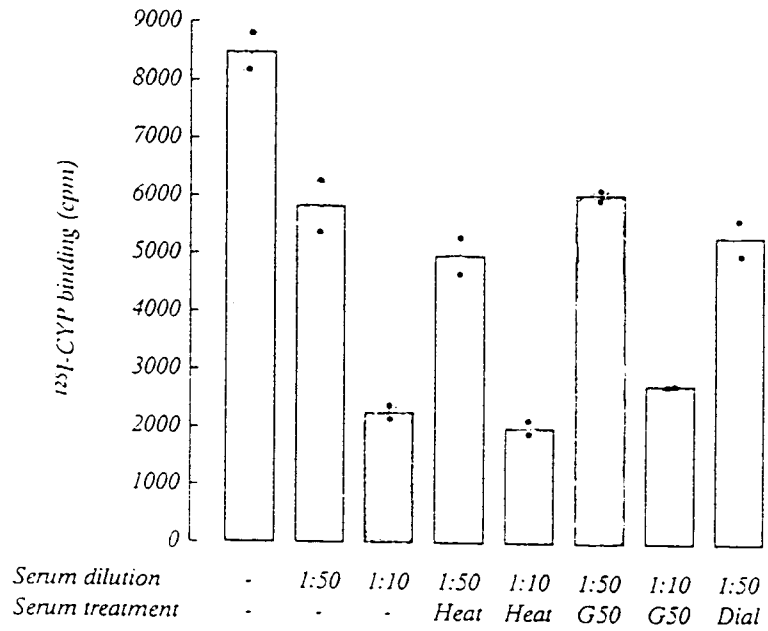


Figure 80. The effect of heat inactivation, dialysis (D) and passing the serum through a Sephadex G50 column on the inhibitory effects of pooled human asthmatic serum on [^{125}I]-CYP binding to cloned human beta-2 adrenergic receptors. Bar values represent mean values of duplicate assays (•).

partially removed by washing the membranes (mean inhibition using 1:50 dilution after washing 26%, unwashed 35%).

I have also attempted to identify fractions in serum containing significant antagonist inhibitory activity after passing 1 ml asthmatic serum over a Sephacryl S200 column. Sephacryl S200 beads were swollen in 50 mM Hepes and the column packed in a 20ml syringe. The flow rate was set at 0.5ml/minute. The outer volume of the column was found to be 8ml (using 1mg/1ml dextran blue in H_2O). A hundred 0.5ml fractions were collected after 1 ml serum was loaded and after freeze drying and reconstitution in 20mM sodium phosphate 2mM $MgSO_4$, one third of each fraction was tested for [^{125}I]-CYP inhibition at the Kd (20pM). Four major peaks of inhibitory activity were identified (Fig. 81). One peak preceded the first protein peak (A), and one peak followed the first protein peak (C) while the other two peaks of inhibitory activity coincided with the major protein peaks.

These experiments indicate that inhibition of [^{125}I]-CYP by serum is complex and serve to confirm that several components in asthmatic (and normal) serum may influence antagonist binding to human beta-2 receptors *in vitro*. Other than investigating the inhibitory activity of purified IgG fractions (6.6.2), I have not attempted to characterise these factors in any further detail. I have observed however that albumin is also inhibitory in the ligand binding assay, in a dose responsive fashion.

In the following experiments I have compared the degree of inhibition obtained by serum when membrane preparations from three transfected B82 mouse cell clones expressing different numbers of human beta-2

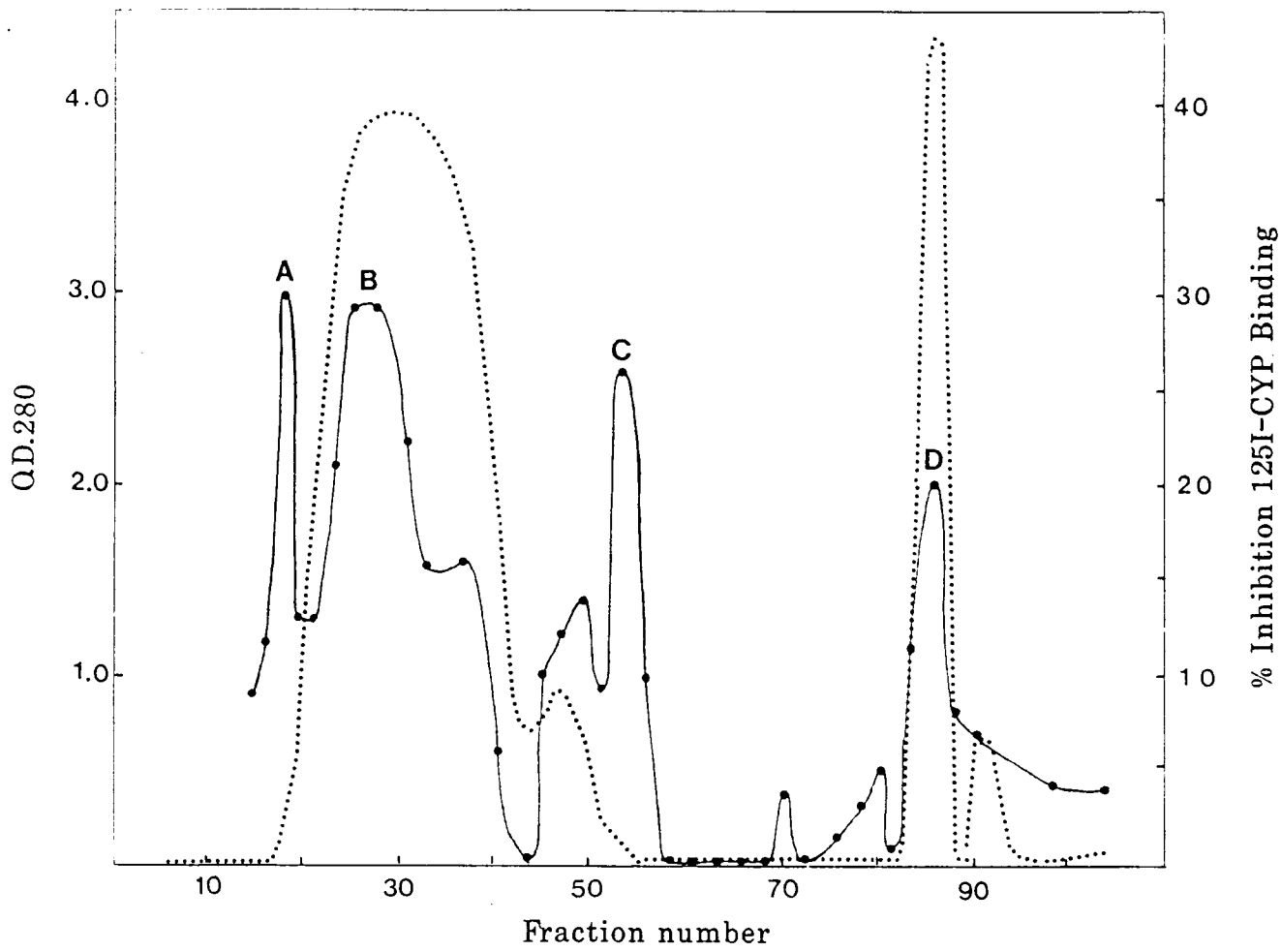


Figure 81. Inhibition of [^{125}I]-CYP binding to cloned human beta-2 receptors by 1:3 dilutions of 0.5ml fractions of pooled asthmatic serum separated on Sephacryl S200 column. OD_{280} of fractions (·····) and % inhibition of [^{125}I]-CYP (——). Peaks A, B, C and D indicate fractions which inhibited [^{125}I]-CYP binding.

receptors (410fmol/mg, 1174 fmol/mg and 4206 fmol/mg) were used. It was quite consistent in repeated experiments that in membranes expressing fewer numbers of beta-2 receptors the inhibitory effect was much greater than in cells expressing greater numbers of beta-2 receptors (Fig. 82). These experiments show that *in vitro* cells expressing lower numbers of beta-2 receptors may be more susceptible to inhibitory effects than others and raise important questions regarding the choice of membrane preparations for [^{125}I]-CYP binding studies and may explain why the degree of serum inhibition I obtained using Wt-7 Cape Town membranes was consistently less than the degree of inhibition I had previously obtained with guinea pig lung membranes.

Although the existence of these antagonist inhibitory factors has been confirmed using guinea pig lung membranes and using cloned human beta-2 receptors, the clinical significance of these factors as applied to human disease remains speculative, particularly since serum from control healthy subjects is also inhibitory.

I have conducted two further studies comparing inhibition of ligand binding to cloned human beta-2 adrenergic receptors mediated by human serum in different groups of asthmatic and control subjects. It is noteworthy that all of the patients whose sera previously inhibited ligand binding to guinea pig beta receptors by greater than 50% (in Table 8) displayed <u>less</u> than 50% inhibition to human beta-2 receptors.

In the first study sera from 14 asthmatic children were compared with 14 non-asthmatic controls and using a 1:50 dilution of serum, no

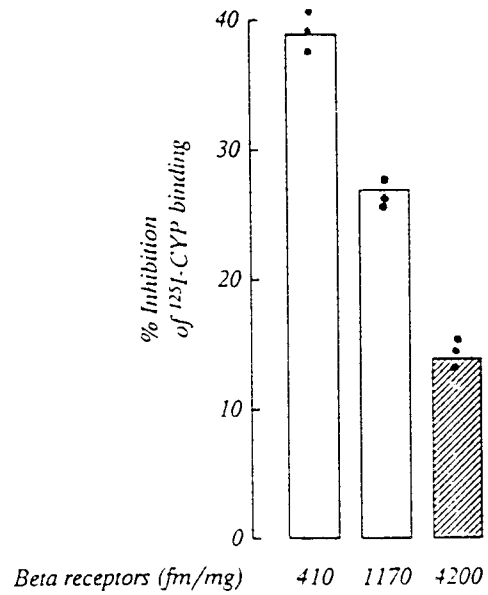


Figure 82. Mean inhibition of [^{125}I]-CYP binding to human beta-2 receptors expressed in membranes prepared from three different transfected B82 clones (410 fm/mg, 1174 fm/mg and 4206 fm/mg human beta-2 receptors). At each assay point there was 10μg cell membrane protein, [^{125}I]-CYP: 20 pM (at the kD) and pooled asthmatic serum (1:50 dilution). Bar values represent men of triplicate experiments (•).

significant difference in the mean inhibition of [^{125}I]-CYP binding was observed.

In the second study sera from 13 different asthmatic children was compared with sera from 9 normal control children and 12 normal adults. In this study, serum was studied at a 1:10 dilution. Results are given in the following Table 17.

TABLE 18. Inhibition of [^{125}i]-CYP binding to human beta-2 receptors by human sera (1:10 dil.)

Group	n	[^{125}I]-CYP binding (Mean inhibition %)	S.E.M.	S.D.
A Asthmatic children	13	47	3.24	11.68
B Normal children	9	31	7.27	21.77
C Normal adults	12	52	3.54	12.26

Considering that a 1:10 dilution of sera was used, the range of inhibition was less than that encountered with the guinea pig lung membrane preparations in Chapter 3.

There was a significant difference between the normal children and normal adults ($p = <0.05$). A significant difference between the mean

inhibition of the asthmatic children (Group A) and the normal children (Group B) was also observed ($p = 0.05$ Student's t) but no difference was found between the asthmatic children and normal adult sera. There was as before, a wide scatter in the results obtained with the normal children and a marked overlap in the data was also apparent.

6.6.2 The affinity of DEAE purified IgG on [^{125}I]-CYP binding to cloned human beta-2 receptors

Studies of the effects of purified immunoglobulin fractions on [^{125}I]-CYP binding at different [^{125}I]-CYP concentrations were also repeated using the human beta-2 receptors. Saturation curves of binding of [^{125}I]-CYP to human beta-2 receptors expressed in B82 cells were conducted in the presence or absence of 1:50 dilution of whole serum (from asthmatic patients), an equivalent IgG (DEAE cellulose purified) concentration as the serum (57μg/500μl), 10^{-6}M L(-)propranalol or 20mm sodium phosphate, 2mm magnesium sulphate buffer pH 7.4 alone. Saturation curves and Scatchard plots of the binding data presented in Fig. (83), show that the competition of purified IgG for antagonist receptor binding was non-competitive (i.e. the Kd for [^{125}I]-CYP binding to the receptor was minimally altered, but the maximum binding (Bmax) was reduced by both serum and IgG preparations).

The affinity of the IgG fraction for the beta-2 receptor was determined using competition curves in which increasing amounts of IgG (range 3.8×10^{-7}M - 1.5×10.5^{-5}M) competed for the binding of [^{125}I]-CYP at the Kd of the ligand (20pM). Using the Prussoff

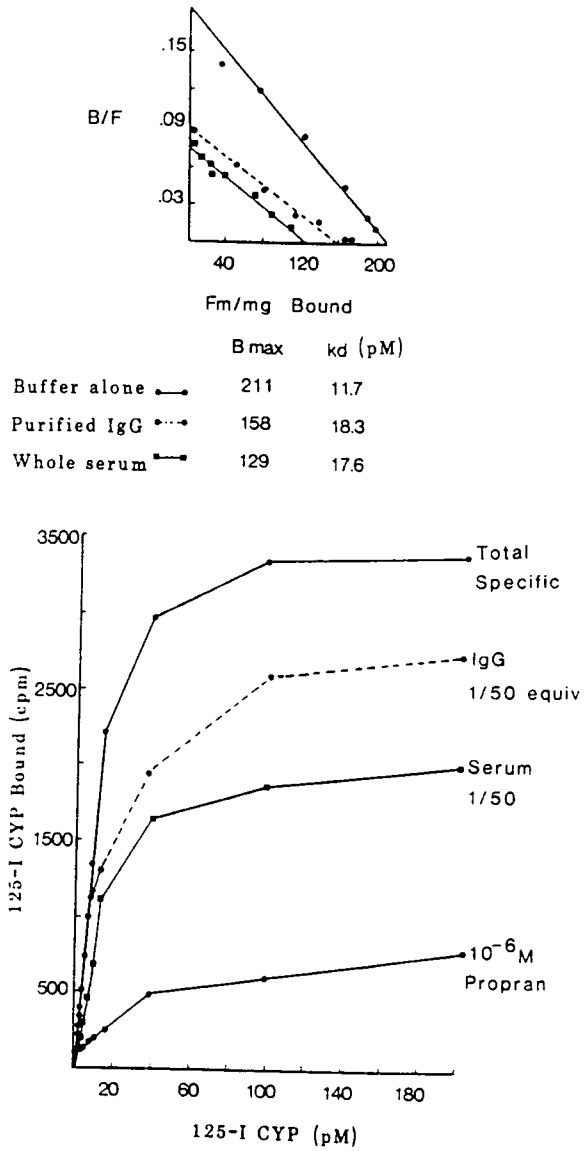


Figure 83. Saturation curves and Scatchard plots of [^{125}I]-CYP binding to cloned human beta-2 receptors in the presence or absence of asthmatic serum (1:50 dilution), purified IgG (equivalent IgG to 1:50 dilution of serum : 57μg/500μl), or $10^{-6)}$M (-) propranalol.

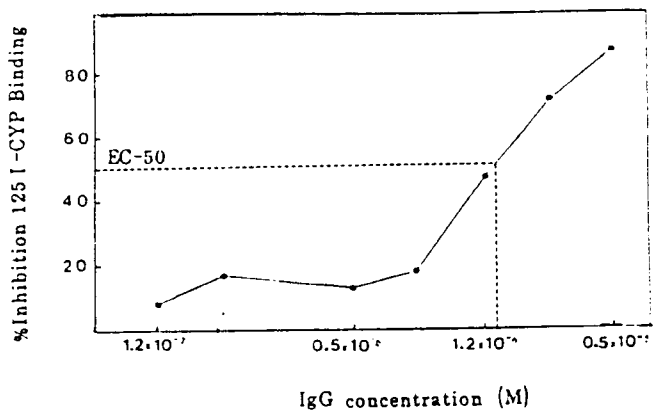


Figure 84. Competition curve showing % inhibition of binding of [^{125}I]-CYP to cloned human beta-2 adrenergic receptors by dilutions (0.5 x 10^{5}M to 1.2 x 10^{-7}M) of DEAE cellulose purified IgG. Using the Prussof equation the affinity of the IgG was calculated to be 4.6 x 10^{-7}M. The concentration of [^{125}I]-CYP in the assay was 32pM.

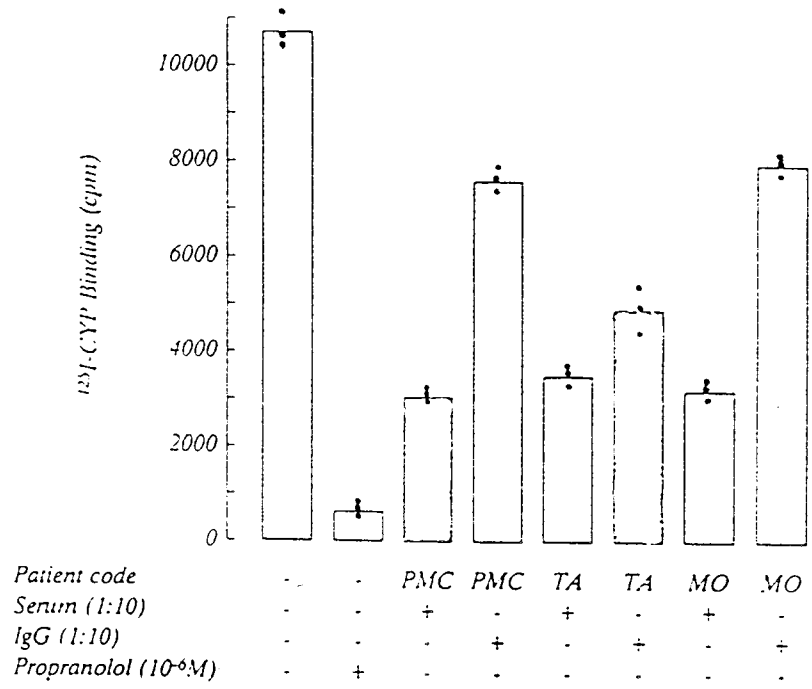


Figure 85. Comparison of the inhibition of [^{125}I]-CYP binding to cloned human beta-2 receptors in Wt-7 Cape Town membranes by dilutions of whole serum (1:10) or equivalent concentrations of DEAE cellulose purified IgG.
Bar values represent the mean of triplicate experiments.

equation, the affinity of the IgG fraction was calculated to be 4.6 x 10^{-7}M. (Fig. 84) (of the same order as isoproterenol).

I have repeated IgG inhibition assays using IgG purified from a further 18 asthmatic patients using membranes expressing cloned human beta-2 receptors. IgG was studied at concentrations equivalent to serum IgG (1:50 dilutions). The IgG fractions were dialysed against sodium phosphate buffer before reconstitution in assay buffer. Representative results from three patients studies are given in Fig. 85 and show again that up to 50% of the total serum inhibition may be exhibited by the IgG fraction, but that other factors also play a role. There was again no difference between the mean inhibition exerted by serum or IgG from asthmatics (Mean = 26; SD = 10.4) and controls (Mean - 21.3; SD = 9.1) p = 0.24 (Kruskal Wallis test).

6.6.3 Summary of the nature of the inhibitory effects of human sera on antagonist ([^{125}I]-CYP) binding to guinea pig and human beta-2 receptors.

1. Both normal and asthmatic sera contain factors which show a dose responsive inhibition of [^{125}I]-CYP binding to guinea pig and human beta-2 receptors (3.2)(6.6.1).
2. There was no significant difference in the inhibition mediated by sera from asthmatics when compared with normal individuals: Kruskal Wallis test p = 0.287 (3.2.2)(although weakly significant differences could be shown using the Student's t tests).

3. Inhibition was not removed by dialysis or heat inactivation (56°C for 30 min) i.e. heat stable (3.7)(6.6.1).

4. Pre-incubation of the membranes with serum/IgG did not increase the degree of inhibition (3.3.4).

5. Significant and, in some cases, equivalent inhibition could be mediated by DEAE purified IgG fractions of the patient's serum, but bore no relation to the total IgG level. (3.3) Although inhibition was mediated by the immunoglobulin fraction, other serum fractions were also found to be inhibitory (6.6.1).

6. Direct antibody binding to guinea pig beta-2 receptors could not be demonstrated on Western blots (3.5) but heterophile antibodies to mouse antigens were found in asthmatics and non-asthmatics (6.4.7).

7. Inhibition mediated by IgG was non-competitive (as shown by Scatchard analysis and saturation curves) and the affinity of the IgG fraction mediating inhibition was 4.6×10^{-7}M (by Prussoff equation)(6.6.2).

8. The degree of [^{125}I]-CYP inhibition mediated by patient serum is relatively constant over a period of time (months)(3.3.1).

9. The degree of [^{125}I]-CYP inhibition is directly related to the number of beta-2 receptors expressed in the cells studied (6.6.1).

6.7 Discussion, and Conclusions.

The expression of cloned human beta-2 receptors in a cellular system has enabled me to verify and extend my studies on the effects of human serum on antagonist [^{125}I]-CYP binding and to conduct original studies on the effects of human serum on agonist mediated cyclic AMP production.

A cell line stably expressing high levels of human beta-2 adrenergic receptors is useful for studying functional effects of serum and cellular components on human beta-2 adrenergic receptors *in vitro*, particularly when receptors expressed following transfection couple with the G proteins and adenylate cyclase. Although successful cell lines were constructed by Chung et al. (1988) the levels of beta-2 receptors expressed in the clones which they characterised were relatively low.

The Wt-7 Cape Town clone provides at a high level of expression a stable source of human beta-2 receptors which bind the ligand [^{125}I]-CYP with high affinity (Kd ±20 pM). Different levels of beta-2 receptor expression in the other clones I obtained permit studies of the effect of receptor numbers on cAMP release.

The level of receptor expression in Wt-7 Cape Town clone far exceeds the levels of beta-2 receptor expression encountered in the physiological state (e.g. in human fibroblasts, myoblasts or

lymphocytes) and it is likely that a considerable percentage of the receptors in Wt-7 Cape Town cells are redundant with respect to their involvement in coupling with the G proteins and adenylate cyclase. Venter (1978) has previously addressed the concept of "spare" receptors under physiological conditions.

I have found that the number of receptors expressed per mg membrane protein has a direct effect on the result of the [^{125}I]-CYP inhibition assay (6.6.1). This observation has important implications for the standardisation of [^{125}I]-CYP ligand inhibition assays and may explain why other workers have found it difficult to obtain reliable and reproducible data using membranes derived from mammalian lungs. Even using guinea pig lungs, I have found that the number of receptors per mg membrane preparation is highly variable. These observations suggest that depending on the system chosen, (cells or tissues), the assay conditions should specify the number of receptors, rather than the amount of membrane preparation (in micrograms), as has been stated by previous workers (Fraser et al. 1981, Blecher et al. 1984). It follows that the use of cell lines rather than whole organs as a source of homogeneous membrane preparations will also improve reproducibility of ligand inhibition assays.

By transfecting a given cell with different numbers of beta-2 adrenergic receptors it may be possible to determine a critical or optimal number of beta-2 receptors required for maximal receptor mediated second messenger (e.g. cAMP) release in that particular cell.

If, physiologically, an excess of beta-2 receptors is expressed on a given cell, this may have certain advantages: for example a receptor reserve will permit a flexibility in the numbers of receptors inactivated, blocked by inflammatory mediators or internalized before a reduction in maximal cAMP release would occur. This concept is important when attributing any possible *in vivo* functional significance to serum or inflammatory factors which are found to block beta receptors in *in vitro* systems. For receptors which are critical for life, a modest reserve would be expected to have a survival value.

Conversely, if a cell constitutively expresses an excess of the optimal number of beta-2 receptors necessary for maximal catecholamine stimulation, the functional significance of further receptor expression e.g. induced by steroids may be negligible.

My own data (6.2.4), comparing maximal cAMP release in cells expressing lower members (~400fmol/mg) of human beta-2 adrenergic receptors, with high numbers (4000fmol/mg) suggested that an increase in cAMP is not linearly related to the level of receptor expression. However, to draw firm conclusions about this observation it will be necessary to study different clones expressing low numbers of receptors which are within the range of physiological expression. Thus, genetically engineered beta-2 receptor expression systems with a high level of expression, while being of great value as a source of beta-2 receptors for purification purposes and antibody binding studies, may not be ideal for functional studies of receptor

activation. Little is known about the critical numbers of receptors necessary for optimal cAMP responses for different cells.

The Wt-7 Cape Town cell line thus has certain advantages and disadvantages in any study of the effects of serum or cellular factors on the function of beta-2 receptors. In the first instance, by using this line, it is clear that for a beta-2 receptor related effect of the serum factor to be detected, by a reduction of cAMP release, a substantial reduction/blocking may be necessary at the receptor level (6.2.4). Thus while the Wt-7 Cape Town cellular system may lack sensitivity in any study of inhibitory factors, a reduction in isoproterenol stimulated cAMP release must imply a significant inhibitory effect of the factor studied, if it can be proven that the effect is, in fact, mediated at the level of the beta-2 receptor.

There are, however, further limitations in studies of functional inhibition in a coupled system, since the inhibitory factor may also block the receptor mediated response at a level distal to the receptor (e.g. G protein or catalytic unit) or by disturbing membrane function non-specifically by e.g. complement lysis (6.4.8). Complement should probably be inactivated before any studies of serum or cell function are conducted. It is difficult, if not impossible therefore, to attribute an effect to the beta-2 receptor level unless specific effects are also studied at G proteins (using sodium fluoride or non-hydrolysable analogues of GTP) or directly on the catalytic adenylate cyclase unit (e.g. using forskolin).

Serum and plasma is a complex mixture of catecholamines, stimulatory and inhibitory factors and while studies of inhibitory factors such as prostaglandins by Omini et al. (1989) and Raaijmakers et al. (1989) in isolation, are informative in experimental models, under physiological conditions mediators are not released in isolation from mast cells and other inflammatory cells. During inflammation serum/plasma factors and cellular activation products are released as a "soup" which may contain a mixture of factors which stimulate adenylate cyclase (e.g. lymphocyte activation products 6.5.3) or inhibit adenylate cyclase (e.g. superoxides, prostaglandins or membrane destructive enzymes released by neutrophil activation). This may be accompanied by increased serum levels of catecholamines and other membrane active substances (e.g. platelet activating factor) which *in vitro* have been shown by Agrawal and Townley (1987) to non-competitively block ligand binding. While it is thus useful to study, in isolation, the effects of purified individual factors released during inflammation on beta-2 receptor coupled adenylate cyclase activation, it would be foolish to attribute pathophysiological significance to the results of such experiments unless the concentration of the factor being studied, its release and its stability, in the local microenvironment in the presence of other mediators, was also known.

The ligand binding studies reported in this chapter were an extension of the studies by Venter et al. 1980, Blecher et al. 1984 and my own studies (Chapter 3) with guinea pig lung membranes. I have demonstrated that dilutions of human sera have a dose responsive inhibitory effect on antagonist [^{125}I]-CYP binding to cloned human beta-2 receptors. On

analysis of [^{125}I]-CYP inhibition studies in 6.6.1 there was consistently a large overlap between the [^{125}I]-CYP inhibition found in normals and asthmatic subjects, and thus the clinical significance of these findings in relation to disease, if any, remains speculative. I have followed up after three years, the asthmatic patients whose sera previously inhibited the [^{125}I]-CYP binding to guinea pig lung membranes by greater than 50% (in 3.2.2) and re-tested their sera. Using membranes from the Wt-7 Cape Town cells, all of the sera inhibited ligand binding by less than 50% (6.6) in the follow up studies. Clinical follow-up of these patients showed a clinical course which was indistinguishable from the other steroid-dependent asthmatics whose sera demonstrated lesser degrees of inhibition in the guinea pig lung membrane ligand binding assays. I could not attach any particular clinical significance to the fact that their sera at a given point in time displayed inhibitory effects in an *in vitro* [^{125}I]-CYP binding assay. However, detailed pulmonary function tests of small airway function and reversibility were not conducted.

In view of the presence of heterophile antibodies to mouse antigens in the sera of many of the patients studied, the functional effects of fresh sera on beta-2 receptor mediated adenylate cyclase activation could not be studied using the fresh sera from patients. Thus to investigate possible functional inhibitory effects of human serum on beta-2 receptors, a human cell line may be preferable and complement must first be inactivated. It is noteworthy that EDTA or heparinised human plasma was not inhibitory at all and this suggests that other than the release of local inhibitory mediators (e.g. PAF) or

neutrophil products, it is unlikely that asthmatics have in their serum factors which are markedly inhibitory for agonist mediated cAMP release.

Future studies, taking these factors into consideration, must be conducted before any hypothesis that asthmatic sera may play a modulating effect on beta-2 receptor function, during inflammation, can be rejected completely. My own studies suggest that complement activation does not play a role in *in vitro* inhibition of antagonist binding, since [^{125}I]-CYP inhibition was not removed by heating at 56°C for 30 min. (3.7, 6.6.1). Since residues in the ligand binding domain important for antagonist binding differ from those involved in agonist binding (and coupling with the G proteins), it is possible that the serum factors influencing agonist and antagonist binding may differ. It is clear that serum factors which do not even directly bind to the beta-2 receptor may disturb its function, if these factors act at the receptor level and at the post-receptor level. Since administration of beta-2 receptor antagonists (e.g propranalol) is contra-indicated in asthmatics, the possible significance of the serum effects on *in vitro* antagonist binding is thus extremely theoretical.

The finding of antibodies recognising mouse proteins in the allergic patient's sera provided a possible explanation for the marked inhibitory effect of the serum on mouse but not human cells. The ability to mount heterophile antibodies to mouse antigens has not previously been studied in great detail. Since both non-allergic and allergic children were found to have heterophile antibodies,it is

probably not linked to the increased facility of allergic asthmatic individuals to respond to other environmental allergens. In a separate study unrelated to the beta-adrenergic receptor I have recently documented (Potter et al. 1991) that the clustering of immune responses to environmental allergens is not random. Whether there is any link between allergen specific immune responses to environmental allergens and heterophile antibody responses in allergic individuals would be interesting to study in the future.

One of the original reasons for embarking on these studies on the beta-2 adrenergic receptor was to investigate the concept of autoantibodies to the beta-2 adrenergic receptor in allergic subjects. Although I have consistently documented that IgG fractions from allergic and non-allergic subjects can inhibit [^{125}I]-CYP binding to guinea pig and human beta-2 adrenergic receptors (with an affinity of the order of 4.6×10^{-7}M)(6.6.2), whole human IgG and $F(ab)_2$ fragments were not found to inhibit cAMP production in functional assays with any of the sera tested (6.5.1). I have not been able to demonstrate any functional effects of these "auto-antibodies" (as defined by Fraser 1981) on agonist binding. Thus before clinical significance of these autoantibodies can be postulated, one should consider the following questions: Are the "autoantibodies" simply an *in vitro* observation which has no real *in vivo* or clinical counterpart, or do immunoglobulins in fact interact with cell membrane receptors as part of a normal physiological process as postulated in 3.7? If one reconsiders the assay conditions for demonstrating [^{125}I]-CYP inhibition, it is apparent that the incubation mixtures used in the

assays of Fraser (1981) and Blecher (1984) and indeed in this thesis do not have a physiological *in vivo* counterpart.

Although I could not demonstrate any antibody mediated blocking effects on cAMP production in my patients, I raised rabbit polyclonal antibodies (701) to the beta-2 receptor peptide (6.5.2) which were significantly stimulatory, supporting the concept that certain antibody containing serum fractions may, under certain circumstances, facilitate cAMP release. Since the rabbit polyclonal antibody 701 also inhibited [^{125}I]-CYP binding to the cloned human beta-2 receptors, it is tempting to speculate that it had intrinsic sympathomimetic activity but this stimulatory activity could have been exerted at a site distal to the receptor.

With the current knowledge of inflammatory factors influencing beta-adrenergic function, the original theories of an inherent or constitutional beta adrenergic receptor dysfunction in asthma should be adapted to accommodate a theory of an acquired beta adrenergic dysfunction but should bear in mind that the degree of inflammation in the airways has not yet been correlated with beta adrenergic hyporesponsiveness in the airways *in vivo*.

Cells which require active catecholamine stimulation for normal airway function (i.e. smooth muscle cells, mast cells, ciliated epithelial cells) are not normally exposed to significant concentrations of serum, plasma or products of activated inflammatory cells. Endogenous catecholamines are thus free to diffuse from the intravascular

compartment into the tissues, unimpeded, to activate the beta-2 receptors, thus effecting and maintaining normal airway smooth muscle tone, normal cilial motility and mast cell cAMP levels. Marked susceptibility of asthmatics to the bronchospasm when given beta blockers such as propranalol, supports a hypothesis that asthmatics are critically dependent upon unopposed catecholamine stimulation.

In the presence of airway inflammation, whether induced by diffuse viral infection, noxious stimuli (e.g. sulphur dioxide) or aeroallergens (i.e. via mast cell degranulation) an increase in vascular permeability permits inflammatory factors to move into the tissues. Inflammatory factors block the activation of beta-2 receptors on these cells leading to adrenergic-cholinergic imbalance, bronchospasm, impairment of ciliary function and may possibly further facilitate mast cell degranulation by making them more "twitchy" as a result of a fall in intracellular cyclic AMP.

As the inflammation subsides, inhibitory effects are reduced and the cells will respond to circulating adrenaline. Since asthmatic subjects' catecholamine responses to stress (or exercise) are blunted (Ind et al. 1985) reduced effector responses would be compounded in the presence of airway inflammation. Steroids or therapy with sodium cromoglycate which reduces airway inflammation, will assist endogenous catecholamine stimulation of the beta-2 receptors and reduce the requirement for exogenous beta-2 stimulatory drugs. Drugs such as ketotifen (Koshino et al. 1988) which also prevent down regulation of

beta-receptors may be of greater benefit if high doses of exogenous beta-2 stimulators are given.

The concept of an acquired but reversible dysfunction in beta-2 receptors in allergic asthmatics is attractive because it explains why asthmatics vary in their requirements for additional beta-2 stimulators and why some mild asthmatics only need beta-2 stimulators during acute respiratory tract infections, or during exercise, or following allergen exposure.

In vitro evidence that inflammation results in reduced beta-2 receptor function has been presented by Raaijmakers et al. (1989), who studied the effects of PAF, LTB4 and LTC4 on [^{3}H]-DHA binding to human lung membranes obtained from thoracotomy specimens and on cAMP release from human lymphocytes. While LTB4 and LTC4 induced a decrease in ligand binding to beta adrenergic receptors to 66.8±14.8% and 71.1±1.4% of control values and PAF reduced the number of high affinity sites to 56.4±8.3% of control values. In the lymphocyte studies basal, isoproterenol and Gpp(NH)p stimulated cAMP release were all decreased after pre-incubation with PAF, LTB4 and LTC4.

The degree of inhibition of antagonist binding reported in these studies is within the range of the inhibition of antagonist binding I obtained with dilutions of normal sera. Their data also suggested that inhibition of cAMP was mediated at a post-receptor or 'membrane' level. Raaijmakers et al. (1986) previously demonstrated that mediators such as PAF not only reduced the number of receptors but also influenced

the affinity of the receptors for agonists. However, it is not known if concentrations of the mediators used in these studies are achieved in the lungs *in vivo*.

Agrawal and Townley (1987) in studies of the effect of PAF on beta-2 receptor function showed that a sub-threshold dose of PAF 0.1μM significantly reduced the potency of isoproterenol to reverse methacholine or histamine induced contraction. They could not demonstrate any change in the affinity of [^{3}H]-DHA binding to human lung membranes induced by PAF, but suggested that the down regulation of beta adrenoceptors was mediated through specific PAF receptors and that a direct interaction with the beta receptor was unlikely. Since PAF has been reported to induce activation of phospholipase A2 and phospholipase A2 has been shown to decrease the density of beta-adrenoceptors on guinea pig lung membranes Taki et al. 1986, it was suggested that activation of phospholipase A2 by PAF could be a mechanism underlying PAF induced down regulation of beta-adrenoceptors.

Kramer et al. (1986) has shown that incubation with 1mM cumene hydroperoxide causes a reduction in the number of beta receptors in lung membrane preparations. Engels et al. (1986) have shown that pulmonary macrophages induce a reduction in beta-adrenoceptor responses of guinea pig tracheal smooth muscle which can be blocked by catalase and thio-urea (an OH. scavenger), suggesting that H_2O_2 and/or OH. may mediate this effect. Rhoden and Barnes (1989) found that responses of tracheal smooth muscle to isoprenaline were unaffected by pre-treatment with H_2O_2 and have suggested that other inflammatory

mediators released by macrophages, in addition to free radicals, may be required to induce deterioration of β-adrenoceptor function. Omini et al. (1989) presented data to support a role for prostaglandins in the beta-adrenoceptor desensitization observed after antigen challenge in ovalbumin sensitized guinea pigs. Again it is not clear whether this effect was mediated at the receptor or at a post-receptor level.

Another consequence of inflammation on beta-adrenergic receptor function should be considered following the studies of Mahan and Insel (1984) who found that catecholamines are susceptible to oxidation when incubated under physiological conditions with intact cells or membrane preparations, and that superoxide dismutase (10μg/ml), but not catalase (10μg/ml) was very effective in inhibiting the oxidation of 10nM isoproterenol. It is thus likely that during inflammation endogenous catecholamines are prevented from diffusing from the circulation to the beta-2 receptors since they are inactivated by free oxygen radicals and H_2O_2.

Evidence that allergen challenge and mediator release reduced beta-2 receptor function/number on circulating lymphocytes has been presented by Meurs et al. (1982, 1984) and recently confirmed by Oehling et al. (1990).

It is widely agreed that airway hyper-responsiveness is a primary abnormality in most asthmatic subjects (Oehling et al. 1990) but the relationship between beta-2 receptor dysfunction and airway hyper-responsiveness is not clear. If, during periods of

inflammation, cellular beta-2 adrenergic receptors are partially blocked, reduced in number or affinity, sequestrated within the membranes, or directly inactivated by bacterial toxins or free radicals and circulating catecholamines are unable to reach their receptors, because they are inactivated by oxidation products of inflammation, this may lead to the cholinergic-adrenergic imbalance favouring bronchospasm leading to an acute attack. If this process is sustained by further mast cell mediator release and cellular recruitment and further damage to airway cells by eosinophils or by viruses, this may lead to a more chronic or severe asthma attack.

It is a general clinical observation that drugs which block the cholinergic system (e.g. atrovent) have not found general application in the treatment of asthma unless combined with a beta-2 adrenergic stimulator (e.g. Duovent). Thus adequate adrenergic drive elevating cellular cAMP levels is essential for the restoration of airway tone.

Since beta-2 stimulators remain the mainstay of treatment of patients with an acute asthmatic attack, this suggests that a certain functional reserve of beta-2 receptors must be present in the airways of even the most severe asthmatics and that any dysfunction induced by products of inflammation must be partial. This, once again, raises an intriguing and critical question which is, as yet, unanswered i.e. how many beta-2 receptors on a given cell can be inactivated before functional effects result? In the genetically engineered system (6.2.4) in which I have obtained cell lines expressing different numbers of human beta-2 receptors, in the absence of inflammation a

large reduction in receptor number did not result in a proportionate reduction in cAMP production. Studies which document the critical numbers of intact beta-2 receptors required on cells of the airway before functional effects of beta-2 receptor blockade are observed would indicate the margin or reserve available in a given patient. It is not known whether this margin is reduced in asthmatic patients. If the beneficial effects of beta-2 receptor stimulation are mediated entirely by the cyclic AMP second messenger, it is possible that other mediators e.g. PGE_1 may, via their stimulation of adenylate cyclase, compensate for partial beta-2 receptor blockade on the same cells. Thus the overall detrimental effect of the inflammatory "soup" of mediators may be less than predicted from the concentration of factors which are known to specifically block beta-2 adrenergic receptors.

In the past, studies to prove or disprove the beta-adrenergic theory of asthma have been fraught with difficulties. The studies which have looked at beta-2 receptor number in membranes of circulating cells e.g. lymphocytes and granulocytes (Brooks et al. 1979, Galent et al. 1980, Sano et al. 1983) in asthmatics, have been done in a serum-free assay buffer, thus excluding any possible influence of the patients serum on the ligand binding obtained. If differences in individual sera can indeed affect the functional ability of their beta receptors by virtue of state of activation of lymphocytes or other mediators, when assessing receptor number in any tissue, the modulatory effects of physiological (pathological) fluids surrounding the receptor and local catecholamine levels must also be considered. It is possible that the reductions in beta receptors reported in the lungs of

asthmatic models of the guinea pig are secondary to non-competitive blocking of the receptors by inflammatory factors in the airways following activated mast cell or ovalbumin sensitization.

Although a primary beta-2 receptor defect at the level of the gene or at the protein level has not yet been identified in any human disease, it remains a theoretical possibility that point mutations similar to those described in Chapter 4 may occur spontaneously in human beings and in asthma particularly, and may induce or contribute to the severity of cardiovascular or respiratory pathology. The effects of such an abnormality may be amplified if "acquired" factors such as mediators of inflammation are superimposed on the genetic abnormality.

Although direct gene sequencing would be the definitive way to identify the occurrence of spontaneous mutant beta-2 receptor expression in human disease, an alternative approach would be to identify and study variants of the receptor identified by restriction fragment linked polymorphisms (RFLP) in patients with a given disease e.g. asthma. As an initial investigation into beta-2 receptor polymorphism in asthmatics, I have studied the distribution of the Ban 1 RFLP in Cape Town allergic and non-allergic subjects and have presented the results of these studies in Chapter 7.

CHAPTER 7

AN INVESTIGATION INTO THE BAN 1 RESTRICTION FRAGMENT LENGTH POLYMORPHISM OF THE HUMAN BETA-2 ADRENERGIC RECEPTOR GENE IN ALLERGIC SUBJECTS.

7.1 Introduction

Acquired alterations in beta-2 adrenergic receptor numbers and function are readily explained by homologous or heterologous regulatory pathways (Stiles 1984). Exposure of beta-2 adrenergic receptors to inflammatory factors from asthmatic subjects *in vitro* may be accompanied by depressed adenylate cyclase responses to isoproterenol stimulation (Chapter 6) and serum mediates non-competitive inhibition of antagonist ligand binding (6.6.2). The possibility that allergic individuals express a structurally abnormal beta-2 receptor, which facilitates or aggravates the beta-2 receptor abnormalities described in allergic asthma, remains an intriguing possibility and has not previously been studied.

Since the majority of asthmatics appear to have normal beta-2 receptor responses to catecholamines between attacks in the absence of on-going airway inflammation and true asthma does not usually develop in the first few years of life (in the abscence of e.g. para influenzae or respiratory syncitial virus chest infections), it would appear that the chance of finding a significant structural abnormality in the

beta-2 receptors of allergic subjects would be small and any structural abnormalities are likely to have a subtle effect on agonist binding or function. In terms of the clinical expression of allergic diseases, it is as yet unknown why some allergic patients develop asthma, some develop rhinitis or eczema while others express combinations of these clinical entities. It is reasonable to speculate that if any of the known allergic diseases were to be associated with a structural abnormality in the beta-2 adrenergic receptor that asthma (in which beta-2 receptor dysfunction has been previously reported) would be the most likely candidate.

The gene for the beta-2 adrenergic receptor and platelet derived growth factor has been mapped to the q31-q32 region of chromosome 5, while in the mouse both genes map to chromosome 18.

The most direct method of identifying a structural genetic defect in the beta-2 adrenergic receptor in allergic individuals would be to construct cDNA libraries from an allergic individuals and to clone and sequence their beta-2 adrenergic receptor genes directly. Alternatively, the polymerase chain reaction may be used to identify abnormalities in the coding region of the beta-2 adrenergic receptor in allergic individuals.

An alternative approach to the identification of a genetic basis for beta-adrenergic dysfunction in allergic disease would be to identify genetic polymorphic variants of the beta-2 adrenergic receptor which occur with greater frequency in allergic individuals, and to study the

molecular nature and function of the proteins coded by such genetic variants.

Since the cloning and sequencing of the human beta-2 receptor genes by Chung et al. (1987) and Kobilka et al. (1987), Lentes et al. (1988) have reported a biallelic restriction fragment length polymorphism of the human beta-2 receptor gene in a study of unrelated North American Caucasians, using the enzyme Ban 1, employing a full length 2.6kb probe of the beta-2 receptor gene. It was remarkable that although over 50 restriction enzymes were studied, polymorphism was only detected with Ban 1 and (less convincingly (personal communication,Ulrich Lentes), with Ava-1).

These experiments suggested that the beta-2 adrenergic receptor gene sequence is highly conserved in man. To detect a polymorphic variant which is specific for allergic asthmatics and not seen in normals (non-allergic individuals), DNA from allergic asthmatic individuals should ideally be screened again for polymorphisms, using the 50 restriction enzymes shown to be non-polymorphic in normal individuals by Lentes. The financial demands of such a study, however, precluded this approach.

I have carefully examined the polymorphic data reported by Lentes and was impressed with the observation that while individuals homozygous for the upper (A) allele (3.7kb), and heterozygotes for both alleles (3.7kb and 3.4kb) were identified, no individuals with homozygous lower alleles (B) were identified. This could simply be attributed to

the rather small population studies (20 individuals), but did suggest that individuals with homozygous lower allele were uncommon. It would thus be interesting to determine the frequency of this homozygous 3.4kb lower allele in other population groups (non-American) and in particular in the allergic population.

In assigning possible functional significance to the Ban 1 polymorphism, it is important to define the site of the polymorphism. A polymorphic site within the coding region could imply a structural difference (i.e. amino acid substitution) in the expressed receptor protein (suggestive of receptor isoforms), whereas a polymorphic site identified in the 3 prime or 5 prime flanking regions of the gene may be compatible with allelic alterations in receptor susceptibility to regulation by glucocorticoids, or to differences in susceptibility to other promoter signals. Both of these possibilities are intriguing possible mechanisms for beta-adrenergic dysfunction in asthma.

In this short chapter, I have studied the allelic frequencies of the beta-2 receptor gene as defined by Ban 1 polymorphism in South African allergic asthmatic and rhinitic children and compared them with non-allergic subjects. I have also compared the frequency of Ban 1 polymorphic alleles in South African children with the North American frequencies (Lentes 1988). I have extended the original studies of Ban 1 polymorphism using the unique 326bp probe of the beta receptor gene sequence which I constructed (Chapter 4), in an attempt to define the site of the Ban 1 polymorphism in the human genomic sequence.

7.2 DNA probes.

Two human beta-2 adrenergic receptor gene probes were used in the polymorphic studies:

(a) A 2.6kb DNA fragment of the human beta-2 adrenergic receptor originally derived from a Pvu II digest of the human genomic clone LCV 517 (containing the entire coding region plus 1000bp of the 5' flanking region and 400bp of the 3' untranslated region of the gene) was obtained by digesting the plasmid PMSG containing the 2.6kb insert (a gift from Dr. Lentes, Bonn FRG) with NheI and XhoI.

(b) A 326bp Kpn-1 - Pst-1 digest of the coding region of the beta-2 adrenergic receptor gene (see 4.2.5). Ban 1 restriction sites and genomic sequences identified by the two probes are illustrated schematically on Figure 86. The 2.6kb probe was labelled by nick translation (Amersham) and the 326bp probe was labelled using a multiprimer DNA labelling kit (Amersham 1601Y).

7.3 Patients' DNA

DNA for the polymorphism studies was prepared from 72 South African individuals.

30 samples were from normal healthy individuals with no history of asthma, allergy or allergic rhinitis. 42 samples were from children over the age of 5 yrs attending the Allergy Clinic at the Red Cross

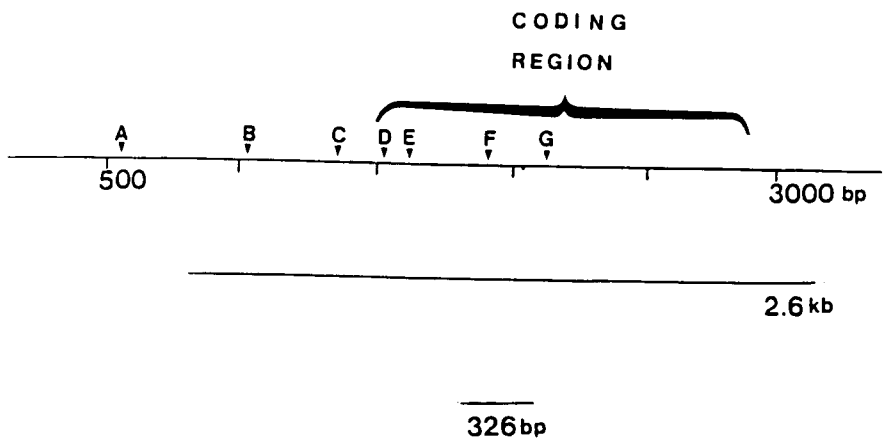


Figure 86. Distribution of Ban 1 restriction sites (designated A-G) in a 3500bp genomic sequence of the human beta-2 receptor gene (coding region and flanking regions). Relationship between the 2.6 kb (Pvu II) probe and the 326bp (KpnI-PstI) probe to the genomic sequence is shown.

Childrens Hospital. All of these children had documented allergies to one or more environmental allergens as documented by skin testing or RAST. 22 of the patients were asthmatics requiring regular bronchodilators and/or steroids, but who had minimal or no clinical evidence of allergic rhinitis. The other 20 patients had severe allergic rhinitis with no asthma.

10ml peripheral blood in EDTA was homogenised in 30 ml lysis buffer (0.32M sucrose, 0.005M $MgCl_2$ 0.01M Tris, 1% Triton X-100 pH 7.6) using a dounce homogeniser (8 strokes up and 8 strokes down), transferred to 50ml polypropylene tubes and centrifuged at 1250 rpm in a Beckman GPR bench top centrifuge at 4°C for 15 min and the pellet washed twice with 20ml of phosphate buffered saline. The pellet was then resuspended in 900μl of STE (Tris 0.01M, NaCl 0.10M and EDTA 0.001M pH 8.0) and 100μl 10% SDS solution added to give a final concentration of 1% SDS. 2-4mg of Proteinase K (Sigma) was added and the solution incubated for 16 hours at 56°C.

To this sample an equal volume of salt-saturated phenol was added and after tumbling for 10-15 minutes centrifuged at 15000 rpm in the microfuge at 4°C. The upper layer was transferred to a clean Eppendorf tube using a pipette which has had its tip cut to prevent shearing of the DNA and after tumbling for 15 min in an equal volume of chloroform, isoamyl alcohol (24:1 v:v) it was centrifuged at 15000 rpm for 15 min.

The aqueous layer was then transferred to a clean Eppendorf tube and DNAse-free RNAse (BRL) added to a final concentration of 50μg/ml and incubated for one hour at 37°C. After repeating the phenol and chloroform:isoamyl alcohol extractions, DNA was precipitated by adding 1/10 volume of 3M sodium acetate pH 6.0 and 2.5 volumes of absolute alcohol. After standing at room temperature for 10 min DNA was spooled out with the end of a Pasteur pipette, washed in 80% ethanol, transferred to a tube containing 200μl distilled water and allowed to dissolve overnight at 4°C. DNA concentration was determined by measuring the optical density at 260nm.

7.4 DNA digestion and Southern Blotting.

5μg human genomic DNA was digested for 6 hours with 20 units Ban 1 (New England Biolabs) at 37°C in 50mM potassium acetate, 20mM Tris acetate, 10mM magnesium acetate, 1mM DTT, pH 7.9 (at 25°C). After denaturing at 65°C for 10 min, DNA fragments were separated in 1xTBE pH 8.0 on 0.8% agarose gels to resolve the restriction fragments. λDNA digested with Hind III was used as a molecular weight marker, and the gel was allowed to run overnight at 30mV until the bromophenol blue dye had moved 12.5-13cm from the origin. The gel was stained with ethidium bromide (1.25μg/μl) for one hour, transilluminated, molecular weight markers photographed and distance the dye front had moved was measured. After denaturation in 1M NaOH for one hour the gel was washed in 3xSSC and Southern blotted on to Hybond N using to the method of Davis (1986) and ethidium bromide staining of molecular weight markers measured under UV light on Hybond N. Blots were

irradiated under UV light for one min prior to prehybridization for 6 hr in 50% formamide, 5x SSC, 2x Denharts Solution, 20mM Na phosphate, pH 6.5, 200μg/ml denatured salmon sperm DNA and 0.1% SDS. The blot was then washed once with 30ml 5xSSC and hydridised for 24 hours at 42°C in a rotating perspex chamber using the [^{32}P]-labelled probe (which had been denatured by boiling for 10 min followed by immediate cooling on ice). After washing the blots twice for 20 min in 2xSSC, 0.1% SDS, the nylon membrane was washed twice in 0.1% SDS, 0.1xSSC at 65°C for one hour, dried between two sheets of Whatman 3mm, wrapped in Saran wrap (to keep it damp) and placed in an X-ray X-omat cassette with an intensifying screen exposed to Kodak AR-5 film at -70°C for 48-72 hr.

7.5 Results

7.5.1 Biallelic polymorphism of the beta-2 adrenergic receptor gene in allergic and non-allergic individuals.

Autoradiographs of human genomic cDNA digested by Ban 1 and probed with [^{32}P]-labelled 2.6kb probe revealed two clear polymorphic loci, 3.1kb and 2.9kb. Homozygotes and heterozygotes for both loci were identified in the allergic and in the non-allergic individuals (Fig. 87). Distribution of these alleles in a family study was compatible with the expected inheritance of these alleles within a family.

A comparison of the frequency of the distribution of the 2.9 and 3.1kb polymorphic alleles on normal (non-allergic) individuals, allergic rhinitis patients, and allergic asthmatic patients is given in Table

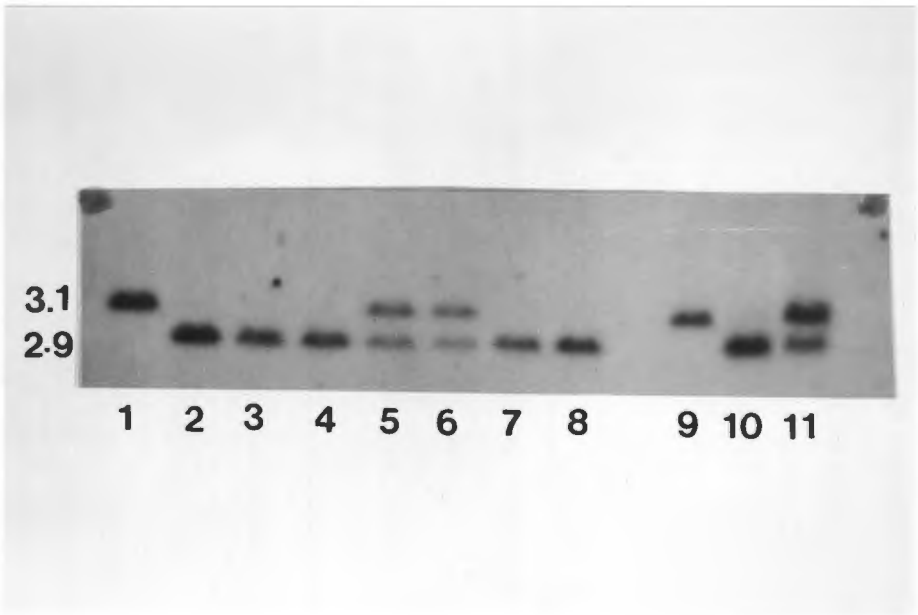


Figure 87. Representative autoradiograph of Southern blot of Ban 1 digested DNA (5μg) from allergic (lanes 1, 5, 6, 8, 9, 10, 11) and non allergic (lanes 2, 3, 4, 7) individuals probed with [^{32}P] nick translated 2.6kb cDNA probe. Molecular weights (kb) are shown on the left. (Stringency 0.1% SDS 0.1xSSC 65°C for 1 hr. Exposure Kodak AR-S film -70.C for 48 hours).

19. The 3.7 and 3.4 kb polymorphic loci identified by Lentes (1988) using the enzyme Ban 1, were not found in any of the 72 patients studied.

Table 19. Distribution of Ban 1 Polymorphism of the human beta-2 adrenergic receptor.

Group	Number	Heterozygous	Homozygous		
			Total	Upper 3.1kb band	Lower 2.9kb band
A.Normal individuals	30	15(50%)	15(50%)	6	9
B.Allergic Rhinitis	20	7(35%)	13(65%)	6	7
C.Allergic asthmatic	22	9(41%)	13(63%)	9	4
Allergic (B+C)	42	16(38%)	26(61%)	15	11
All individuals	72	31(43%)	41(56%)	21	20

Analysis of the relative frequencies showed no significant difference between the allergic and non-allergic groups with respect to the frequency of this polymorphic locus. Gene frequencies are shown in Table 20.

Table 20. Gene frequencies of beta-2 adrenergic receptor polymorphic alleles.

A. South Africans:	Gene Frequency (Upper 3.1 allele)	(Lower) 2.9 allele
Group A:		
Normal non allergic individuals	0.45	0.55
Group B & C:		
Allergic Individuals	0.45	0.55
Group B:		
Allergic Rhinitis	0.47	0.53
Group C:		
Allergic Asthma	0.61	0.39
B: North American Subjects (Lentes 1988)	0.85	0.15

The occurrence of homozygous 2.9kb and homozygous 3.1kb alleles in the same frequency in both normal and allergic individuals suggests that neither of these polymorphic forms are specifically associated with the expression of the allergic phenotype.

7.5.2 Defining the site of Ban 1 polymorphism of the human beta-2 receptor gene

It was in the first place important to confirm that Ban 1 in fact cut human DNA at the predicted restriction site, i.e.

G ↓ G(T)(A)C C

(C)(G)

C C(A)(T)G ↑ G

(G)(C)

I have located the Ban 1 restriction sites of a 3000bp genomic sequence of the human beta-2 receptor gene containing coding region, as well as three prime and five prime sequences (Fig 86). Seven potential Ban 1 sites are present, designated A-G. It is evident that Ban 1 cuts the genomic DNA under consideration into fragments less than 700 bp. Sites B-G lie within the gene sequence spanned by the 2.6kb cDNA probe. Restriction fragments as large as 2.9kb and 3.1kb must lie three prime to site G (which lies within the coding region of the gene) if they are detected by the 2.6kb cDNA probe.

To confirm the predicted restriction sites I have also digested purified 2.6kb cDNA fragment with Ban 1. An autoradiograph of a 1.0% agarose gel showing the Ban 1 digested fragments of the 2.6kb probe, probed with 2.6kb probe (Fig. 88) confirmed that the majority of the fragments were less than 700bp. A fragment ± 1.7kb and undigested 2.6kb

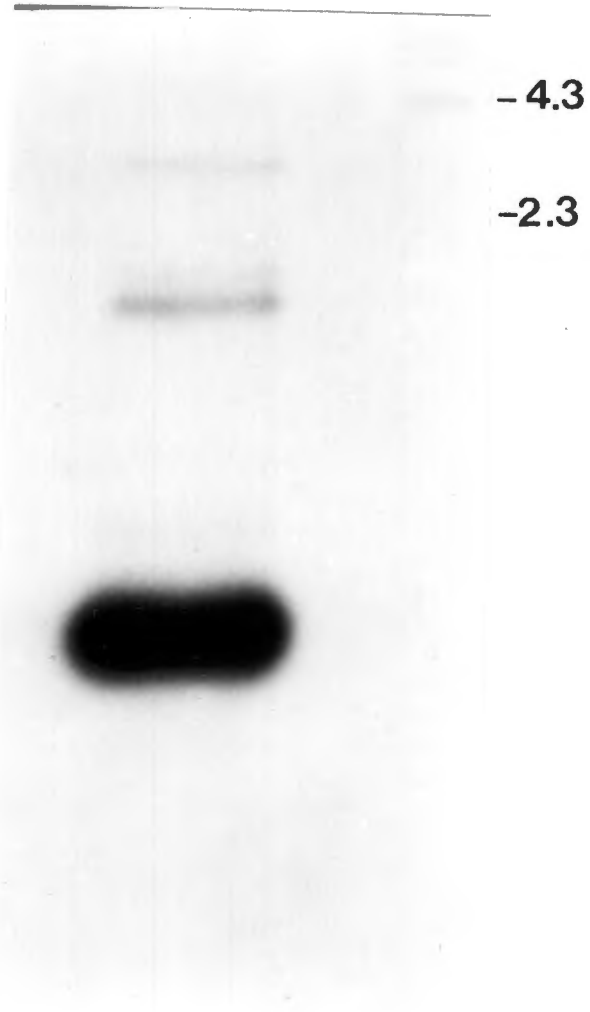


Figure 88. Autoradiograph of the 2.6kb genomic DNA probe after digestion with Ban 1. The restriction fragments observed are in accordance with the predicted fragments in Fig. 86. Most of the 2.6kb genomic probe is digested into small fragments (less than 700bp), but a 1.7kb fragment and undigested 2.6kb probe is also seen.

residual fragments are also clearly shown in the same figure and confirms that the Ban 1 restriction sites do occur as predicted from the restriction map. These findings suggest that the 2.9 and 3.1kb polymorphic gene fragments must include residues which lie three prime to the 2.6kb probe.

Extending these arguments, I reasoned that if the polymorphic site was at site G, a probe spanning sites F-G would only detect the 3.1kb (larger) allele. The Kpn-1-Pst-1 326bp probe which I constructed in 4.2.5, corresponds almost exactly to the F-G fragment. I therefore probed DNA from patients who were shown to be heterozygotes (using the 2.6kb cDNA probe) with the random primer labelled 326bp probe and found that only the larger fragment was identified (Fig. 89). These findings support a hypothesis that a polymorphic locus exists within the coding region of the beta-2 adrenergic receptor gene.

To confirm this hypothesis and strengthen the above conclusion, it would be useful to construct probes which only recognise sequences of the beta-2 receptor which lie five prime to the Ban 1 site F. Probes recognising these sequences should not identify the 3.1 or 2.9 kb restriction fragments.

7.6 Discussion

The experimental evidence I have presented in this chapter confirms that both allergic and non-allergic individuals display a Ban 1 polymorphism of their beta-2 adrenergic receptor gene. In the first

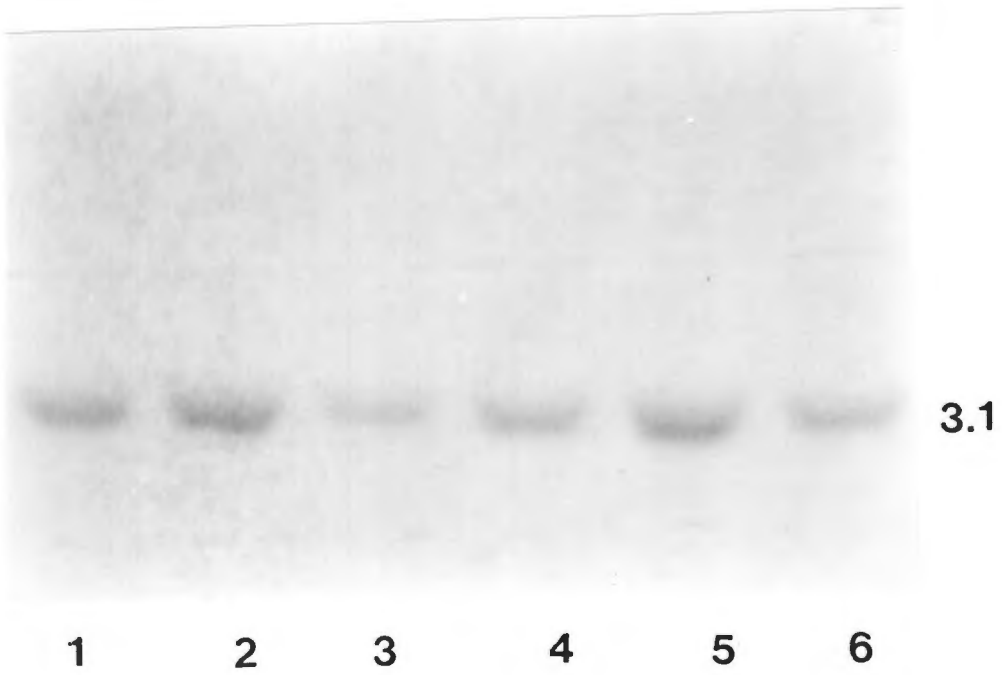


Figure 89. Autoradiograph of human genomic DNA digested with Ban 1 previously shown to be homozygous or heterozygous using the 2.6kb probe and reprobed with random primed 326bp probe after stripping the 2.6kb probe. Only the larger allele, 3.1 kb was detected with the 326bp probe. Lanes 1-3: DNA from Cape Town subjects. Lanes 4-6: DNA from West German subjects (from Dr. K.U. Lentes). Lanes 1,3,4 and 6 were heterozygous and lanes 2 and 5 were homozygous for the larger allele.

instance the 2.9kb and 3.1kb fragments I consistently obtained were distinctly smaller than the 3.4 and 3.7kb fragments reported by Lentes (1988). I therefore obtained DNA from three additional patients which Lentes confirmed were upper band homozygous or heterozygous for the larger or the smaller allele, (on his return to Bonn, West Germany), and found that the 3.7kb allele of Lentes corresponded to the 3.1kb allele on my autoradiographs and the 3.4kb allele of Lentes corresponded to the 2.9kb allele on my autoradiographs. Furthermore, the 320bp probe only identified the larger allele of the same size, shown on a single gel, both in the DNA I had prepared and the DNA prepared by Lentes in Bonn (on Fig. 89).

In contrast to the findings of Lentes, I also found several normal and allergic patients who were homozygous for the lower (2.9kb) allele. I had no explanation for the differences in size of the restriction fragments but Lentes (personal communication) has conceded that he may have over-estimated the size of the two fragments he identified.

Since both polymorphic forms are found with equal frequency in the allergic population and in normal control populations, it would seem reasonable to conclude that both polymorphic forms are associated with normally functioning beta-2 receptors.

Individuals homozygous for the 2.9kb allele presumably have Ban 1 site G intact (Fig. 86) whereas individual homozygous for the 3.1kb allele would have disturbed Ban 1 cutting at site G. The amino acid coded for at site G is amino acid 177 (Tryptophan) and substitution of tryptophan

with another amino acid could theoretically produce certain conformational changes is the receptor, but since this occurs in normal individuals as well, the functional effects of a genetic change at this site on the receptor expressed and on ligand binding or function appear to be minimal.

Thus one would conclude that the Ban 1 restriction polymorphism is probably not an important genetic factor in the expression of the allergic phenotype.

It would of course be interesting to compare in more detail the function of beta-2 adrenergic receptors from allergic and normal individuals who are homozygous for the 2.9kb or 3.1kb alleles in further studies.

The overall gene frequency for the 2.9kb allele is 0.49 and for the 3.1kb allele is 0.51, in the 72 South African patients studied. This clearly differs from the North America studies in which the gene frequency of the larger allele was reported to be 0.85 and the smaller allele 0.15. Thus I have demonstrated a clear population difference for this polymorphism.

Recently several investigators have focussed studies to define the regulatory regions of the human and hamster beta-2 adrenergic receptor gene. The identification of important regulatory sites permits one to hypothesise that point mutations or abnormalities in the five prime or

three prime untranslated regions may result in differences in receptor expression which may lead to clinical disease.

The start of transcription determined for the human beta-2 adrenergic receptor gene is at nucleotide position -219 relative to the start of translation. About 30 base pairs from the start site of transcription are two TATA box-like sequences (-238 and -252) and a reverse complement of the CAAT box located an additional ~50bp upstream (Kobilka 1987). Furthermore, there are also elements in the five prime region which contain consensus sequences for transcriptional regulation by glucocorticoids (Chung et al. 1987) and cyclic AMP. In the human beta-2 adrenergic receptor gene two candidates for glucocorticoid responsive elements include a proximal element at position -248, adjacent to the TATA box (AGTCCTGT,) or a distal element further upstream at position -1447 (TGTTCT)(Collins 1989). Thus, if allergic individuals displayed polymorphisms at the critical regulatory loci, responses to steroids may be impaired. This identified another area for further study possibly using polymerase chain reaction to identify these critical sequences in the allergic population.

From the studies I have completed in this chapter a clear genetic basis for beta-2 adrenergic receptor dysfunction in allergic asthmatic individuals has not emerged. However, the possibilities for investigation of a genetic defect in these patients have not been exhausted. These original studies also represent the first direct examination of the beta-2 adrenergic receptor gene in allergic

subjects. Besides direct gene sequencing of the coding area of the beta receptor, non-coding regions should also be studied in the future.

Postulated mechanisms on acquired beta-2 adrenergic receptor dysfunction in asthma are supported by experimental data in this thesis. Until hard data can be found to confirm the contrary, the theory of a constitutional beta-2 adrenergic receptor abnormality in asthma must remain but a theory.

CHAPTER 8

FUTURE DIRECTIONS

In the study of pathophysiological mechanisms which may explain clinical disease, *in vitro* systems are useful, if direct measurement of the *in vivo* system cannot be performed for ethical or practical reasons.

In the past *in vitro* systems employing membrane preparations, purified receptors, cell homogenates and more recently whole cells have been used to identify factors which could influence beta-2 receptor function *in vivo*. It is important that the *in vitro* system should closely resemble the physiological state if valid comparisons are to be drawn.

In applying published *in vitro* methodology to detect and investigate factors influencing beta receptor function, I have identified a number of disadvantages particularly with the use of the ligand binding inhibition assay.

On first principles, the use of non-human beta-2 receptors in non-physiological membrane preparations to study the effects of human serum on the binding of a synthetic antagonist (e.g. [^{125}I]-CYP), which has a ligand affinity in the picomolar range, can hardly be expected to produce data which would have clinical significance.

My data has confirmed that even after optimising the conditions for receptor viability, ligand binding and reproducibility, the antagonist ligand inhibition assay produces data in which there is a substantial overlap between "patients" and controls (Chapter 3). This problem was not eliminated or improved by using cloned human beta-2 receptors in the ligand inhibition assay (Chapter 6). Thus the value of the [^{125}I]-CYP or [^{125}I]-HYP inhibition assay in providing useful information in patients with asthma is indeed questionable.

An expression system in which cloned human beta-2 receptors are functionally coupled to G proteins and adenylate cyclase in living cells is more physiological and superior to studies in membrane preparations. I have found that although the transfected mouse cells (B82 cells) provided a rich source of human beta-2 adrenergic receptors in a more physiological system, unexpected factors inherent in human sera (e.g. heterophile antibodies) limit the use of this system in the study of the effects of heat labile inhibitory factors in fresh serum on beta-2 adrenergic receptor function.

Factors which act distally to the receptor, (e.g. G protein, adenylate cyclase), or on the cell membrane itself, may also significantly disturb beta-2 receptor function. Thus, in future, it would be important to examine the effects of candidate "beta receptor inhibitory factors" at several levels before functional effects can be attributed specifically to the beta-2 receptor site. Applying a system in which some cells have human beta-2 receptors transfected in them while control cells have no beta-2 receptors (e.g. using non-transfected B82

cells as a control and Wt-7 Cape Town cells) will be most useful in identifying effects which are exerted at a receptor, or post-receptor level.

Using the Wt-7 Cape Town expression system I have identified another area about which little is known, i.e. the optimal level of beta-2 receptor expression for a given cell. It is likely that too high a level of expression of a gene product in a cell, particularly if regulated artificially, (e.g. by a steroid inducible promoter) may not represent a physiological state at all. Even in the non-functional assays, differences in the degree of ligand inhibition were observed with membrane prepartions from B-82 cells expressing different numbers of human beta-2 receptors. In this regard it would be important to document whether steroids do, in fact, increase beta-2 adrenergic receptor number in the lungs of asthmatic patients and whether any increases induced are in fact of functional importance.

I have not been able to show that purified IgG from asthmatic patients (whose sera are inhibitory in the [^{125}I]-CYP ligand binding assay) exerts any functional inhibitory effect in the Wt-7 Cape Town cells. It is possible that larger numbers of patients should be studied, before this possibility can be excluded completely.

Unless human immunoglobulins can be shown to bind to specific extracellular domains or the intramembranous ligand binding pocket in living cells, it is likely that the concept of "autoantibodies to the beta-2 receptor" presents no more than an *in vitro* phenomenon. Milgrow

and Witebsky's criteria for autoimmunity (1962), have not yet been fulfilled for the beta-2 receptor antibodies, and no-one has yet produced a convincing correlation of this phenomenon with a disease process. It would be interesting in the future to investigate whether T cell clones recognising the human beta-2 receptor are found in asthmatic patients (or normal individuals), since this may provide a more convincing basis for a theory of autoimmunity than the current evidence attributed to antibodies.

These studies have also illustrated some of the difficulties in producing a "control" monoclonal antibody which in fact meets the desired specificities. The B4A8-PWL monoclonal antibody did not serve the purpose for which it was produced, but did provide useful indirect information. Since it is highly specific for a 49kDa molecule identified in several cells, it can be used to purify, identify and study this molecule in the future. The relationship between the 49kDa protein and the beta-2 adrenergic receptor (if any) would also be very interesting to explore.

The level of action of the polyclonal antibodies produced in rabbit 701 with cAMP stimulatory activity, should be studied further. While antibodies stimulating beta-1 receptors have previously been identified (Sterin Borda 1984), human antibodies stimulating beta-2 adrenergic receptors directly, have never been identified. The Wt-7 Cape Town cell system could easily be used to look for beta-2 receptor stimulatory antibodies in human sera provided that serum is decomplemented or heat inactivated. Such antibodies could also be

investigated using expression systems developed by others or using lymphocytes.

The role of the inflammatory response in the pathogenesis of asthma has been increasingly emphasised in recent times, and it is highly likely that if an acquired "defect" in beta-2 receptor function develops during the exacerbation of the disease, that this is due to an inflammatory "mediator". My own preliminary studies suggest that products of neutrophil activation may block or inhibit isoproterenol stimulated cAMP release and I have immediate plans to study this in more detail. It will be possible to use the Wt-7 Cape Town system to examine the effects of any mediator or drug on beta-2 agonist stimulated cAMP release in future studies and to study the effects of different levels of receptor expression on the susceptibility to, or resistance to inhibitory effects of inflammatory factors.

My studies of the nine mutant human beta-2 receptors produced data consistent with more recent models of receptor topography in the membrane and contribute to an improved understanding of the overall molecular nature of the receptor, by identifying critical residues for ligand binding and receptor function. By utilising the available knowledge of the beta-2 receptor gene sequence, the effects of steroids and cAMP on gene transcription and the construction of highly specific beta-2 receptor probes, it will be possible to apply genetic techniques to study the regulation of beta-2 receptor expression in human airways, using techniques such as *in situ* hybridization.

To date, a structural defect in the human beta-2 receptor gene sequence has not yet been identified. In my own studies of the Ban 1 polymorphism of the human beta-2 receptor gene, I could find no differences between the allergic patients and non-allergic controls. This, however, can only be regarded as a start to future studies. Mutational studies have identified several residues which are important for normal functioning of the beta-2 receptor. Using these mutational models it will be possible in the future, using the polymerase chain reaction to screen and identify abnormalities in short sequences of these critical residues in patients with disease, without having to sequence their entire beta-2 receptor genes. Thus, it will be no longer necessary to rely on indirect *in vitro* evidence to identify beta-2 receptor abnormalties in human disease, and it will be possible to determine conclusively whether or not a structural defect in the beta-2 adrenergic receptor gene occurs in human disease.

It would be ideal to be able to identify beta-2 receptors directly in patient's lungs using non-invasive methods. Techniques involving scanning or imaging should be developed so that changes in their level of expression and state of activation of beta-2 receptor can be studied in relation to observed pulmonary function tests. Scanning could also be used to assess the effects of, and appropriate requirements for, beta-2 agonist therapy. Developmental models of beta-2 adrenergic receptor expression which show sequential and rapid changes in beta-2 receptors such as I have found in the developing foetal and neonatal guinea pig lung, could be used to test the reliability and validity of non-invasive measurements of receptor number and function when this technology becomes available in the future.

APPENDIX A

Lactoperoxidase iodination of guinea pig lung membranes.

Activity of commercial lactoperoxidase was assessed as follows: Using a PYE UNICAM PU 8800 UV/VIS spectrophotometer, set at 25°C and 350nm, 0.15ml of 90mM H_2O_2 was diluted in 30 ml of 5mM KI in 33mM Na_2PO_4 buffer pH 7.0. 3.0 ml of this reaction mixture is placed in the cuvette and the blank read, after which 0.01ml of lactoperoxidase (1mg/1ml) is added and the increase in absorbance recorded for 3-4 min.

Lactoperoxidase activity was calculated to be 138 units/mg. Glucose oxidase 1200U/ml (Sigma) was diluted to 0.03-0.04U/ml for the assay. Briefly 10μl lactoperoxidase (10U/ml), 10μl glucose oxidase (10U/ml), 10μl glucose (100mg/ml), 10μl Na[^{125}I] and 100μl lung membrane (1.2mg) were stirred for 30 minutes and loaded on to two 1 ml Sephadex G50 columns in two aliquots of 70μl which were centrifuged at 800g for 2 minutes and radioactivity of the eluate counted. 5μl of eluate had 9300 cpm measured on a Packard gamma counter (70% efficiency).

APPENDIX A1

Recovery of iodinated membranes from Sephadex G50 columns.

	Spin 1			Spin 2			Spin 3		
Column	Loaded cpm	Counts recovd	%	Re-loaded	Counts recovd	%	Re loaded	Counts recovd	%
1	11000	6197	56	5552	2867	51	2481	1580	63
2	11000	6385	58	5441	3065	56	2681	1308	51
3	11000	5860	53	5337	2844	53	2466	1338	54
4	11000	5683	51	5193	2802	55	2440	1203	49

APPENDIX B

Preparation of alumina and Dowex columns for adenylate cyclase assay.

Racks accommodating 24 columns each were constructed (by Mr. B. Orlandi) so that they fitted into each other in pairs. The racks held plastic columns 180mm x 6 mm (inner diameter) (Greiner 0.025 ml micropipettes No. 601 502) which were spaced so that the effluent from the lower columns drained into 24 scintillation vials arranged in a standard tray.

Dowex AG 50W-X4 (200-400 mesh)(Biorad) was washed repeatedly with deionised water until the effluent was colour free. A 50% v/v slurry in deionised water was placed in a beaker and stirred vigorously. While stirring, 2 ml samples of homogeneous slurry were added to the glass wool stoppered columns. Columns were filled with water to allow all the beads to settle and form the column bed. Columns were then washed with 2 ml 1N HCl. Prior to use the columns were filled with water (± 20 ml) and allowed to drain.

Alumina Columns: 0.6 g dry neutral alumina (Sigma) was scooped into glass wool stoppered columns, washed with 15 ml 1.0M Imidazole buffer pH 7.3 followed by 20 ml of 0.1M imidazole HCl buffer pH 7.3. Columns were stored at room temperature. Elution volumes for ATP and cyclic AMP were determined using the step by step method described by Salomon (1979). Recovery from the Dowex columns was approximately 93% and from the alumina columns 89% using the [^{3}H] cAMP tracer and [α-^{32}P]ATP.

APPENDIX C

Determination of the optimal concentration of rabbit anti-human IgG for immunoprecipitation.

Human myeloma IgG purified by triple ammonium sulphate precipitation was estimated to be about 95% pure. Purified human IgG was iodinated using the iodogen method published by Thorpe and Johnstone (1978). 1 mg Iodogen was dissolved in 1 ml dichloromethane and dispensed in 20μl aliquots into conical 10.5 x 39mm polypropylene tubes. The tubes were rotated in 37°C waterbath until the solvent evaporated leaving a film of Iodogen at the bottom of each tube. 10μl of 0.25M sodium phosphate pH 7.5 and 10μl purified IgG (1mg/ml) was added to an Iodogen tube and then vortexed. 200μCi sodium iodide (Amersham) was dispensed into the iodogen tube and the reaction mixture incubated for 10 minutes, mixing at 1 minute intervals. Upon completion of the incubation the reaction mixture was loaded on to a 2 ml Sephadex G50 fine column. The 0.5ml eluate was discarded and 6 x 0.5ml aliquots of 0.154M PBS were added to the column and collected separately in polypropylene tubes. 10μl from each tube was diluted 1/10 000 in PBS and counted in a Packard gamma counter. Fractions containing radioactivity were pooled and 10μl 10% albumin added to each fraction. Radioactivity incorporated into the IgG protein was measured by trichloracetic acid precipitation (approximately 30%).

Rabbit anti-human (RαH) IgG was prepared by immunization of New Zealand White rabbits with purified human IgG and tested against unlabelled

human IgG using the Ouchterlony double immunodiffusion technique. Visible precipitation occurred using neat rabbit serum and human IgG neat, 1:2, 1:4 and 1:8 dilutions.

Optimal concentrations for the rabbit anti-human IgG and human serum dilutions was determined by constructing immunoprecipitation curves using [^{125}I]-labelled human IgG as a tracer.

50 000 cpm [^{125}I]-labelled IgG was dispensed into microfuge tubes with 100μl 1:25, 1:50, 1:250 and 1:500 dilutions of pooled human serum, and made up to a final volume of 200μl with 20mM sodium phosphate buffer, pH 7.4. Ten dilutions of rabbit anti-human IgG ranging from 1:10 to 1:500 000 were prepared. IgG concentrations in each human serum dilution were measured using a Beckman Nephelometer. 100μl of RαH IgG was added to [^{125}I] IgG spiked human serum dilutions, incubated overnight at 4°C spun in the Beckman microfuge for 30 minutes and the pellet counted using a Packard gamma counter.

Precipitin curves using three dilutions of rabbit anti-human sera showed that good precipitation could be obtained with human serum at dilutions of greater than 1/50 using dilutions of rabbit serum from 1:3, 1:12 and 1:50. A dilution of 1:12 was chosen for the rabbit antibody in the immunoprecipitation studies (3.4.1).

APPENDIX D

Immunogenicity of lung membrane preparations in the New Zealand white rabbit.

In order to investigate whether it was possible to induce [^{125}]-CYP ligand inhibitory antibodies by exposing one species to intact beta receptors of another, lung membranes with functional beta receptors (confirmed by [^{125}I]-CYP binding assay and adenylate cyclase assay) prepared from human, guinea pig and calf lungs (using the methods described in 2.2.1), were injected into New Zealand White (NZW) rabbits.

Immunization protocol.

For the primary immunization, 200μg lung membranes (human, guinea pig and calf) in 1 ml phosphate buffered saline, emulsified in 1 ml complete Freunds adjuvant was injected on to the dorsal aspect of 3 NZW rabbits in 200μl aliquots. Eighteen days later the rabbits received a booster injection of 200μg of the same lung membranes in incomplete Freund's (IFA) adjuvant. A control rabbit received no immunization. The rabbits received a standard rabbit diet. (EPOL rabbit maintenance pellets).

The rabbits were observed for any clinical sign of disease and baseline blood samples were taken for the measurement of immunoglobulins and serum chemistry.

Course of the immunised rabbit

Four months after primary immunization the rabbit immunised with guinea pig lung membranes died. No environmental cause for the rabbit demise was apparent and a postmortem revealed nothing abnormal in any tissue. Histological examination of the tissues was not performed.

The other two rabbits appeared to be thriving seven months after primary immunization. The rabbit which had received calf lung membranes was boosted again with 200μg calf lung membrane in IFA.

By ten months after primary immunization both rabbits were noted to have developed significant alopecia on the ventral surface and on closer inspection there was loss of muscle bulk and obvious wasting which was more marked in the rabbit immunised with human membranes. Ten ml clotted blood was taken from each rabbit, serum separated and aliquotted and stored at -20°C. The animals were then weighed and examined regularly.

Antibody measurements of 10 months showed that both rabbits were negative for anti-mitochondrial, anti-smooth muscle, anti-nuclear factors and anti-thyroid antibodies but that the rabbit immunised with calf lung membranes had developed a titer of anti-microsomal antibodies of 1:400. Serum (1:50 dil) from the rabbits did not inhibit [^{125}I]-CYP binding to guinea pig lung membranes.

A photograph taken of the rabbit immunised with human lung membranes is shown in Fig. 90 and compared with the normal control rabbit. The wasting observed was striking. The rabbit was weak and apathetic.

The other rabbits in the University of Cape Town Animal House showed no sign of any similar disease problems.

Weights shown for the two rabbits over the next four months were monitored and shown in the following table (weight in grams).
(+ = demise)

	Weight (grams)	
Date	Rabbit immunised with human lung	Rabbit immunise with calf lung
6.12.85	2670	3040
9.12.85	2925	3105
13.1.86	2330 +	3100
15.1.86	-	2715
24.1.86	-	2250
22.4.86	-	2320 +

The weight chart confirms that the rabbits had developed a wasting disease which became rapidly progressive in the rabbit immunised with human lung membranes. Serum chemistry measured on 12.12.85 in both rabbits showed AST, ALT and LDH normal for both rabbits but creatine kinase was elevated in the rabbit immunised with human lung compared with normal rabbit control. The rabbit immunised with human lung died on 13.1.86 and a detailed postmortem was performed. A blood culture on 18.12.85 had revealed Staph. epidermidis sensitive to penicillin and the rabbit was treated with penicillin for one week.

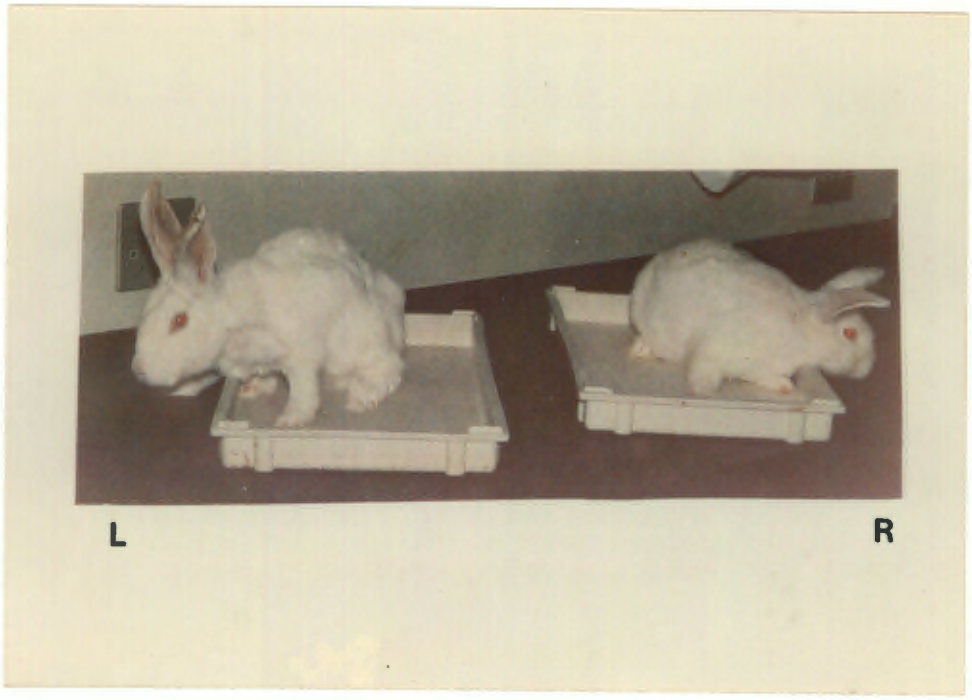


Figure 90. Development of marked wasting in New Zealand rabbit 1333 immunised with human lung membranes (left) compared with a non-immunised rabbit (right).

The rabbit immunised with calf lung continued a slower but a steady downward course. On 29.1.86 serum phosphorus and creatine kinase were noted to be lower and alkaline phosphatase, aspartate amino-transferase and alanine amino-transferase levels higher than pre-immunisation serum levels. Serum proteins, urea and electrolytes were normal but urinary urea was elevated 2.5 times that of the normal control rabbit. Serum from the rabbit again did not inhibit [^{125}I]-CYP binding to lung membranes. The rabbit continued its downward course becoming progressively apathetic and wasted although a small septic lesion was found on the left leg on 29.1.86 and although no organisms were cultured from the septic site the rabbit was treated with a short course of Penicillin. The rabbit died on 22.4.86.

Antibodies raised in the rabbits

An 11% SDS PAGE of 100μg human lung membranes, rabbit lung membranes and crude rabbit lung homogenate and guinea pig lung membrane preparations was immunoblotted (Western blotting technique) on to nitrocellulose paper using a modification of the method originally described by Towbin (1978).

In order to screen for antibodies to injected lung membrane proteins, 1:500 dilution serum from the rabbit injected with calf lung membranes was incubated with a nitrocellulose immunoblot of calf, guinea pig and rabbit lung membranes. HRPO-conjugated goat anti-rabbit antibody and a 4-chloronaphthol substrate was used.

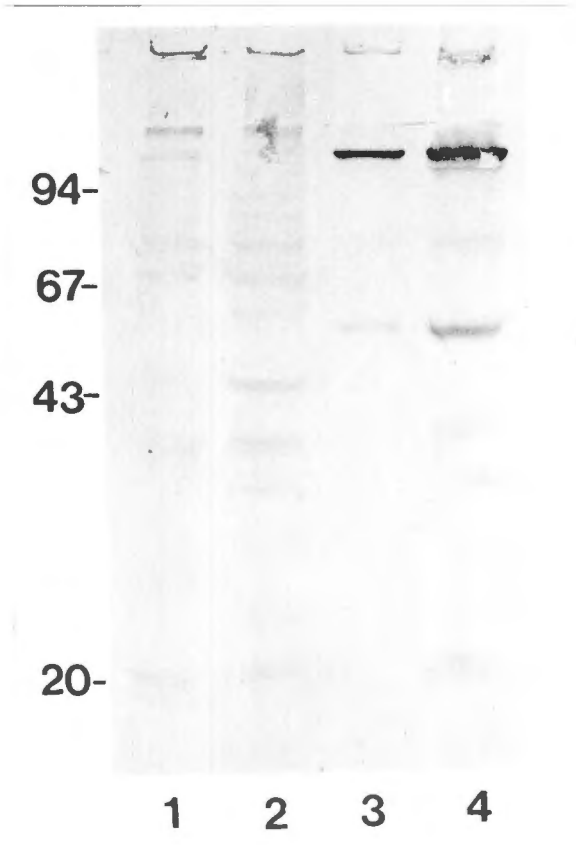


Figure 91. Nitrocellulose immunoblot of calf (lane 1), guinea pig (lane 2), rabbit membrane (lane 3) and crude rabbit lung homogenate (lane 4) incubated with 1:500 dilution of rabbit serum (Appendix D).

As shown in Fig. 91 the rabbit immunised with calf lung membranes developed antibodies to a number of membrane proteins in the calf lung preparation, but also reacted with a number of proteins in guinea pig lung membranes and strongly reacted with high molecular weight rabbit lung membrane proteins.

Postmortem morphology and histology

The postmortem findings and histological examination (performed by Dr. J. Campbell, pathologist) are shown in tabular form as follows:

Table 21: Postmortem findings of rabbits immunised with foetal human and foetal calf lung membranes.

	Rabbit 1333	Rabbit 1335
Immunising agent	Human lung membranes from 16wk human foetus, (containing beta adrenergic receptors)	Lung membranes from foetal calf lung (containing beta adrenergic receptors)
a)Gross pathology	Wasted rabbit with marked ventral alopecia and ulceration of the anterior chest wall	Wasted rabbit with marked ventral alopecia
Lungs	Haemorrhage	Congested
Heart	Normal	large left ventricle
Aorta	Atheroma present	Diffuse yellow mottling atheroma
Liver	Congested	Autolysis
Kidneys sectioning	Cortex yellow	Cortex mottled on
	Large pale kidneys Fatty change in cortical tubules	Normal size
Stomach & gut	Dilated and filled with bile stained material Marked dilatation of caecum	Marked dilatation of caecum
Adrenals	Large and Yellow	Appear normal
Spleen	Soft and gray	Dark and atrophic
Thymus	Atrophic	Small and atrophic
Eye	Nictitating membrane fixed over most of the the orbit	-
Bones	Appear unusually densely calcified	Densely calcified

		Rabbit 1333	Rabbit 1335
Immunising agent		Human lung membranes from 16wk human foetus, (containing beta adrenergic receptors)	Lung membranes from foetal calf lung (containing beta adrenergic receptors)
b)	HISTOLOGY	Rabbit 1333	Rabbit 1335
	Kidneys	A severe and extensive tubular lesion was noted. Most of the renal tubules are replaced by basophilic calcorpherules and the remaining tubules were dilated or atrophic. Calcification also present in Bowmans Capsule and rarely in the glomerular tuft. A striking eosinophilia was noted in the glomeruli. Casts were present in some dilated tubules but vascular changes were not a feature. No evidence of renal infection.	Extensive renal calcification and renal fibrosis of periglomerular tissue tubules and some vessels. Some vessels show degeneration and calcification. Cellular infiltrate is not a feature
	Aorta & vessels	The aorta and its main branches show calcification of the subendothelial muscularis.	Aortic medial degeneration with calcification and intimal fibrosis. Severe medial calcification with intimal fibrosis in the main coronary arteries with calcification of the heart valves and roots of the aorta and pulmonary artery. The left ventricle appeared hypertrophied.
	Lungs	Bronchitis and calcification of the bronchial cartilage.	Focal congestion and haemorrhage with calcification of bronchial wall and basement membrane.
	Skull	Reorganization of lamellar bone with with deposition of amorphous calcium and phosphate around the remaining bony lamella.	Greatly thickened trabeculae and lamellae showing many pagetoid cement lines.
	Spleen	Severe siderosis	Siderosis organ autolytic
	Eye	Oedema and fibroblastic thickening of the iris	Eye and hardens gland autolytic

	Rabbit 1333	Rabbit 1335
Immunising agent	Human lung membranes from 16wk human foetus, (containing beta adrenergic receptors)	Lung membranes from foetal calf lung (containing beta adrenergic receptors)
Uterus	Uterine atrophy	Calcification of meso-metrial and outer myometrial vessels.
Thymus	Involution	
Adrenals	Prominant fascicular zone	thick vacuolated fascicular layer.

Discussion of the pathology of the rabbits

Immunization of three rabbits with lung membrane preparations employing a standard immunization protocol has not only induced an antibody response to proteins in the lung membranes, but in two out of three animals a severe but strikingly similar wasting disease associated with alopecia, renal damage and diffuse generalised calcification.

The observation that antibodies raised to calf lung membrane proteins also bound to rabbit lung membrane proteins and were associated with disease in rabbit, suggest that either identical antigens are present in both species or that the antibodies bind to cross reactive epitopes.

The fact that the effects on the rabbit lungs were small when compared with the dramatic effects observed on the kidneys and vessels, suggests that the antibodies raised in the rabbit may have been directed against antigens in these organs, or deposited there as immune complexes.

The exposure of an animal species to lung membranes from a different species is thus able to induce a wasting autoimmune type of process. Clearly these experiments are preliminary and need to be followed up by

detailed and systematic immunization of further rabbits with lung membranes or homogenates but the similarity of the pathological processes observed in these animals suggests that this protocol may be useful as a model for atherosclerosis in the rabbit.

It is furthermore tempting to speculate that in the human situation, exposure to intact cell membranes from other animals could also be immunogenic and possibly induce autoimmune processes in individuals in whom the regulating mechanisms for antibody production are inadequate. There was no evidence of antibody production to the beta receptor or the development of asthma in the rabbits at any stage.

APPENDIX E

Fibroblast cell lines and culture conditions.

E1 Cell lines

E1.1. B82-L cells

A clonal subline of L cells (B82) lacking thymidine kinase (Littlefield 1966) was used in the studies conducted at N.I.H. (Bethesda, Maryland). These L cells originally derived from the C3H/AN mouse strain, were found to lack catecholamine responsiveness probably due to lack of the human beta-2 receptor or masking of its funtion (Gilman 1973).

For the transfections and studies performed at the University of Cape Town B82 cells cultured from frozen N.I.H. stocks were shipped to Cape Town by Dr. Josephine Lai (University of Arizona, U.S.A.) in tissue culture flasks. These cells were found to be contaminated with mycoplasma and were decontaminated according to the protocol in Appendix E4 prior to the transfections.

E1.2 Foreskin fibroblast line

Human foreskin fibroblasts were a gift from Dr. Colleen Fearns (University of Cape Town). These were derived from frozen stocks of primary cultures from foreskins obtained from neonatal circumcision.

E2 Maintenance and storage of cell lines

Cell lines were maintained in RPMI-1640 (RP; Gibco Cat. No. 074-1800) supplemented with 10% FCS (heat inactivated; 56°C for 30 min) 300μg penicillin/ml and 200μg streptomycin sulphate/ml. The cultures were kept at 37°C in a humid atmosphere containing 5% CO_2 in air in a Hotpack CO_2 incubator (Hotpack Corporation, Philadelphia, PA USA).

Cell lines were examined daily under phase contrast microscopy and passaged when they approached confluency. Passaging was performed by incubating the cells for 5 minutes in 0.25% Trypsin in medium at 37°C. The detached cells were dispersed by gentle pipetting and the suspension added to an equal volume of medium containing FCS to neutralise the trypsin. After centrifugation at 350g for 5 min the pellet was resuspended in medium and re-seeded into culture dishes.

For long term preservation cell stocks were frozen in liquid nitrogen by resuspending a cell pellet in medium containing 10% FCS and 10% DMSO in 1 ml volumes in screw topped 38 x 12.5mm nylon tubes (Greiner Cat. 121 261). Tubes were wrapped in paper towelling at -70°C overnight prior to freezing in liquid nitrogen.

E3 Screening for mycoplasma contamination.

All cell lines used were tested for mycoplasma contamination according to the method of Chen (1977). Cells were fixed with acetic

acid:methanol (1:3) and stained with 0.5μg/ml of bisbenzimide fluorochrome (Hoechst No. 33258) in Hanks balanced salt solution for 30 min. The cell layer was rinsed with deionised water and mounted in buffer containing 0.02M citric acid, 0.06M disodium phosphate and 50% glycerol, pH 5.5. Cultures were examined under a Nikon fluorescence microscope.

E4 Eradication of Mycoplasma Contamination.

The B82 mouse L cells which were obtained from Dr.J. Lai (University of Arizona), were found to be heavily contaminated with mycoplasma, using the Hoechst stain. To eliminate mycoplasma from the cells, I used the BM cycline protocol (Boehringer Mannheim). There was no evidence of mycoplasma contamination after the fourth week of treatment. The B82 cells were checked periodically, using the Hoechst stain and they subsequently have remained free of mycoplasma contamination.

APPENDIX F

Membrane preparation from cells in culture

Cells were grown to semi-confluence in 15 cm tissue culture dishes and pulsed with 10^{-6}M dexamethazone (Sigma Cat. D4902) for 24 hours, to induce beta-2 receptor expression in transfected cells, prior to harvesting. 6ml cold (4°C) lysis buffer 5mM sodium phosphate pH 7.4 2mm $MgSO_4$ was added to each dish and after the cells were scraped off the dish with a rubber policeman, they were left on ice for 30 min. Cells were then homogenised by 20 strokes of a glass homogeniser (Wheaton, USA), centrifuged at 1000g for 10 minutes to remove intact cells and cellular debris. The supernatant was then centrifuged at 40 000g for 45 min at 4°C using a 70Ti rotor in a Beckman L-80 ultracentrifuge to collect the crude membranes. Membranes were suspended at approximately 4 mg/ml in 150μl aliquots in 20 mM sodium phosphate 2mM $MgSO_4$ pH 7.4, snap frozen, in screw capped 38 x 12.5 mm nylon tubes (Greiner) and stored at -70°C.

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Site-directed Mutagenesis and Continuous Expression of Human β-Adrenergic Receptors

IDENTIFICATION OF A CONSERVED ASPARTATE RESIDUE INVOLVED IN AGONIST BINDING AND RECEPTOR ACTIVATION*

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Using a new expression vector that allows stable and steroid inducible expression of the human β_2-adrenergic receptor in mouse L cells, we have examined the functional significance of the highly conserved aspartate residue in the putative second transmembrane region of the receptor. Substitution of aspartate 79 with asparagine produced a mutant receptor that displays the expected affinity and stereoselectivity for antagonists but a 40-, 140-, and 240-fold reduction in its affinity for isoproterenol, epinephrine, and norepinephrine, respectively. This receptor mutant does not display guanine nucleotide-sensitive high affinity binding of agonists. Addition of saturating concentrations of isoproterenol to cell cultures expressing the mutant receptor produces a slight, albeit significant, increase in intracellular levels of cyclic AMP as compared to cells expressing wild type receptor. These observations demonstrate that substitution of aspartate with asparagine at residue 79 in the human β-adrenergic receptor differentially affects the binding of catecholamines and produces a functional uncoupling of receptors and stimulatory guanine nucleotide regulatory proteins (G_s). These data are consistent with a role for aspartate 79 as a counterion to the amine in catecholamines and in agonist-induced activation of the β-adrenergic receptor associated with high affinity ligand binding, G_s coupling, and adenylate cyclase stimulation.

Recent cloning (1) and stable expression (2) of the gene encoding the human β_2-adrenergic receptor in murine cell lines allows for detailed examination of the significance of conserved amino acid residues in receptor function by site-directed mutagenesis. The aspartate residue at position 79 within the second putative transmembrane segment of the human β-adrenergic receptor is part of a sequence domain that is conserved in all β-adrenergic (3-6), α-adrenergic (7), and muscarinic cholinergic receptors (8-10) and all opsin proteins (11-13) sequenced to date. The Asp residue in this domain has been proposed to form a counterion for the protonated Schiff base involved in retinaldehyde binding to opsin (14). Similarly, it has been suggested that this residue may serve as a counterion to the amino group of bound catecholamines in the β-adrenergic receptor (14). Somewhat consistent with this hypothesis were data in a recent report on the substitution of Asp-79 with alanine in the hamster lung β_2-adrenergic receptor (15). A 10-fold loss in receptor affinity for agonists was reported for this transiently expressed mutant receptor. The transient nature of receptor expression precluded a more thorough investigation into the role of Asp-79 in receptor function.

In order to study the function of this important Asp residue in more detail, we introduced a unique point mutation in the human β-adrenergic receptor to change Asp-79 to asparagine and have utilized a new expression vector to obtain stable, glucocorticoid-inducible expression of the mutant β-receptor in B-82 cells, a murine cell line lacking β-receptors (2). Our data suggest that Asp-79 is involved in an agonist-induced activation of the β-adrenergic receptor associated with transduction of a biological signal to other membrane effectors.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from sources described previously (2).

Construction of a New Expression Vector and Mutagenesis—Expression vector pMSVneo was constructed by replacement of the xanthine-guanine phosphoribosyltransferase (GPT) gene in plasmid pMSG with the neomycin-resistance gene from pRSVneo. A new polylinker was also constructed to facilitate the cloning of the β_2-adrenergic receptor gene.

For receptor expression, the entire coding region of the human β_2-adrenergic receptor derived from genomic clone LCV-517 (1) (base pairs −6 to +1267) was cloned into the *Mlu* and *Eco*RV sites in the polylinker region of pMSVneo. The single base mutation that converts aspartate to asparagine was introduced into the human β_2-adrenergic receptor by oligonucleotide-directed mutagenesis according to the method of Kunkel (16). Authenticity of the mutation was confirmed by dideoxy sequencing. Cells were transfected and selected as described by Fraser *et al.* (2).

Cell Culture—B-82 cells, a murine L cell line, were grown in monolayer as described (2).

Cell Membrane Preparation and β-Adrenergic Receptor Ligand Binding Studies—were performed as described by Fraser *et al.* (2). Protein was assayed using the fluorescamine assay with bovine serum albumin as a standard (17).

RESULTS

In order to study the role of specific amino acid residues in neurotransmitter receptor function, we have constructed a new expression vector that allows stable and inducible expression of eucaryotic genes in mammalian cells. As shown in Fig. 1*A*, the expression vector, pMSVneo, contains the 1450-base pair long terminal repeat of the mouse mammary tumor virus. The dexamethasone-inducible transcription promoter within the mouse mammary tumor virus 5′-long terminal repeat regulates expression of genes inserted into the downstream multiple cloning site. Addition of 1 μM dexamethasone to culture medium results in a 5- to 10-fold increase in the density of β-adrenergic receptors in transfected cells (data not

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> (+)-isoproterenol > (−)-norepinephrine.

It is well documented that the affinity of β-adrenergic receptors for agonists is decreased when assays are performed in the presence of guanine nucleotides. This change in receptor affinity is presumed to reflect the dissociation of a "ternary complex" of agonist, receptor, and G_s that is associated with high affinity for agonists (18). In order to examine whether or not the Asn-79 receptor displayed guanine nucleotide-sensitive changes in agonist affinity, competition binding studies with isoproterenol and ICYP were repeated in the presence of 100 μM guanyl-5′-yl imidodiphosphate (Gpp(NH)p) (Fig. 3). In membranes containing wild type receptors, addition of exogenous guanine nucleotides caused a shift to the right in the isoproterenol dose-response curve and eliminated the majority of the high affinity component of agonist binding. The Hill coefficient for isoproterenol binding to wild type receptor was 0.5 in the absence of guanine nucleotides and shifted to 0.8 in the presence of Gpp(NH)p. In contrast, the Hill coefficient for isoproterenol binding to the mutant Asn-79 receptor in the absence of Gpp(NH)p was 1.0, indicating a single homogeneous (low affinity) population of ligand binding sites. Gpp(NH)p had no effect on isoproterenol binding to Asn-79 receptors. These data suggest that mutagenesis of Asp-79 to Asn resulted in a functional "uncoupling" of the G_s effect on the β-adrenergic receptor.

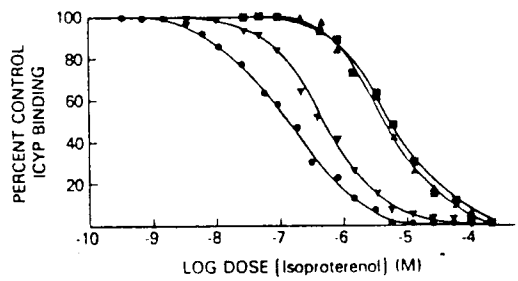

FIG. 3. **Guanine nucleotide regulation of agonist affinity to wild type and mutant β-adrenergic receptors in transfected cells.** ICYP specific binding to wild type and mutant receptors was determined at a concentration of 60 pM in the presence of increasing concentrations of (−)-isoproterenol plus or minus 100 μM Gpp(NH)p. ▼ and ● illustrate the binding of ICYP to wild type receptors in the presence and absence of Gpp(NH)p, respectively. ▲ and ■ illustrate the binding of ICYP to Asn-79 mutant receptors in the presence and absence of Gpp(NH)p, respectively. Samples were assayed and filtered as described in Fig. 1. Data are representative of two separate experiments, each point performed in triplicate.

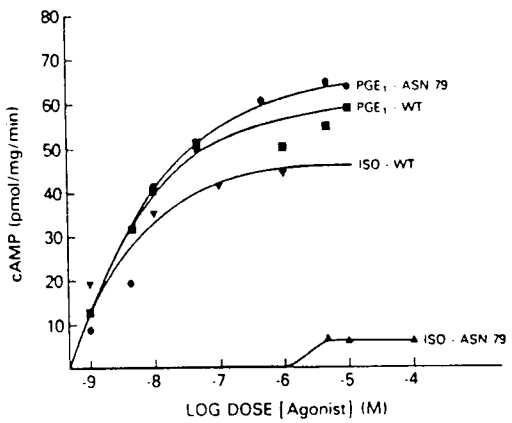

FIG. 4. **Stimulation of adenylate cyclase in cells expressing wild type and mutant Asn-79 β-adrenergic receptors.** Confluent cultures of cells expressing wild type (WT) or mutant (ASN 79) receptors were washed with Dulbecco's phosphate-buffered saline (PBS) and incubated in PBS containing 1 mM 3-isobutyl-1-methylxanthine for 30 min at 37 °C. Isoproterenol (ISO) or PGE_1 at the indicated final concentrations was added to the cultures for 20 min. PBS was aspirated from the cultures and intracellular cyclic AMP was extracted with 2 ml of 6% trichloroacetic acid. Trichloroacetic acid extracts were extracted four times with ether, and cyclic AMP concentrations were determined using a radioimmunoassay kit for cyclic AMP (Du Pont-New England Nuclear). Cells were dissolved in 0.2 N NaOH for determination of cellular protein. Data are expressed as picomoles of cyclic AMP produced per milligram of cellular protein per minute.

The ability of isoproterenol to stimulate adenylate cyclase in cells expressing Asn-79 β-adrenergic receptors was investigated. As we previously reported (2), addition of isoproterenol to B-82 cells containing wild type receptor resulted in a dose-dependent increase in intracellular levels of cyclic AMP similar to those elicited with PGE_1 (Fig. 4). In contrast, addition of increasing concentrations of isoproterenol to cells expressing mutant Asn-79 receptors had no significant effect on intracellular levels of cyclic AMP except at the highest isoproterenol concentrations (10–100 μM) where slight but statistically significant increases in intracellular levels of cyclic AMP were seen in cells expressing Asn-79 receptors. This increase in intracellular cyclic AMP was blocked by the concomitant addition of (−)-propranolol (10 μM) to cell cultures, indicating that the observed changes in cyclic AMP levels were mediated by a receptor specific activation of adenylate cyclase (data not shown).

DISCUSSION

In order to study the role of specific amino acid residues in β-adrenergic receptor function, we have substituted aspartate 79 with asparagine. The locus of this mutation is within the second putative transmembrane domain of the β-receptor. This alteration produced a mutant β-receptor that displayed normal binding of antagonists but a reduced affinity for agonists. In addition to the observed change in receptor affinity for agonists, replacement of Asp-79 uncoupled the G_s effect on receptor. While Asn-79 mutant receptor no longer displayed guanine nucleotide-sensitive binding of agonists, agonist activation of adenylate cyclase was still observed, albeit at significantly attenuated levels.

These data raise the question as to the role of aspartate 79 in normal β-adrenergic receptor function. Receptor modeling studies predict that aspartate 79 is located in the aqueous core formed by the hydrophilic surfaces of the seven amphipathic transmembrane segments of the receptor,[2] and therefore, it is unlikely that this amino acid directly interacts with G_s.

A more likely hypothesis is that aspartate 79 is involved in maintaining a receptor conformation necessary for high affinity agonist binding and transfer of this binding energy to other effectors. Thermodynamic studies of agonist interactions with β-adrenergic receptors were consistent with a two-step process of agonist binding and an associated conformational change in the receptor (17, 18). As proposed by Applebury and Hargrave (14), Asp-79 may serve as a counterion to the amine group of the catecholamines in the β-adrenergic receptor. Replacement of a negatively charged aspartate residue with an asparagine having no net charge may eliminate one point of catecholamine attachment to the receptor, resulting in a loss of agonist affinity. The differential loss of agonist affinity observed in the Asn-79 mutant receptor may reflect differences in hydrophobic interactions between ligand and receptor at this locus. For example, the isopropyl substituent of isoproterenol and the methyl group of epinephrine could provide differential hydrophobic interactions that sta-

[2] A. R. Kerlavage, R. J. Feldmann, and J. C. Venter, manuscript in preparation.

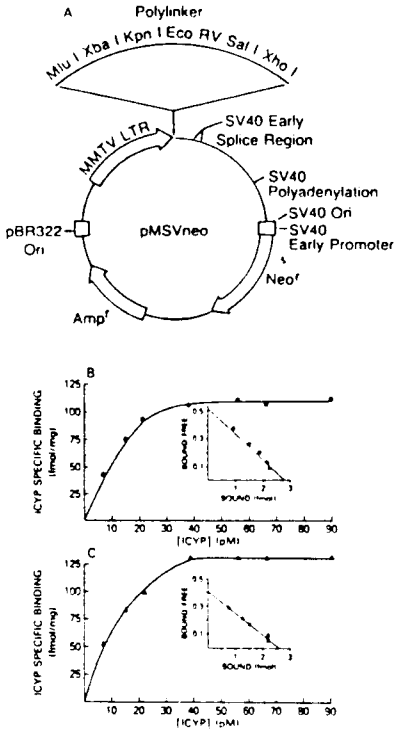

FIG. 1. **Mutagenesis and expression of human β_2-adrenergic receptor gene in transfected B-82 cells.** *A*, expression vector pMSVneo. *B*, saturation isotherms of ICYP binding to membranes from cells expressing wild type human β_2-adrenergic receptor. Membranes from cells pretreated with 1 μM dexamethasone for 24 h before harvesting were incubated with the indicated concentrations of ICYP in the presence or absence of 1 μM (−)-propranolol for 30 min at 30 °C. Samples were filtered over Whatman GF/C filters using a Brandel M-24R Cell Harvester and washed with 20 ml of phosphate-buffered saline. Data are representative of two separate experiments, each point performed in triplicate. Nonspecific binding was between 5 and 10% of total binding. *Inset*, Scatchard analysis of ICYP binding to wild type β-adrenergic receptors (19). *C*, saturation isotherms of ICYP binding to membranes from cells expressing Asn-79 mutant β_2-adrenergic receptors. Assays were performed and analyzed as detailed in *B*.

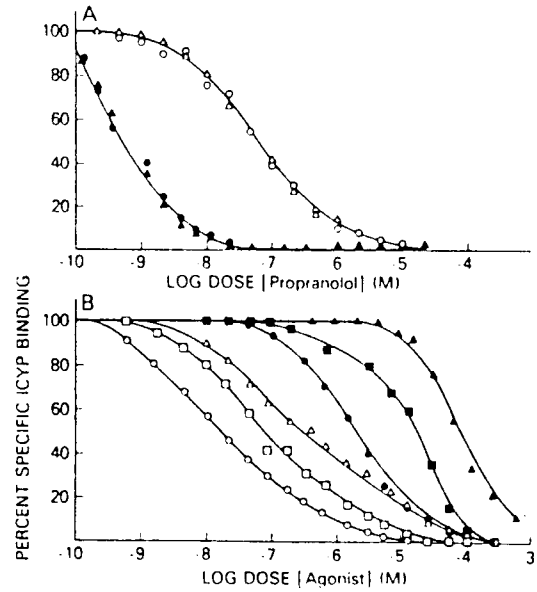

FIG. 2. **Adrenergic agonist and antagonist competition for ICYP binding to expressed human β-adrenergic receptors.** Membranes from dexamethasone-treated cells expressing wild type or Asn-79 mutant β-adrenergic receptors were incubated in the presence of 10 pM ICYP and the indicated concentrations of adrenergic agents for 30 min at 30 °C. Samples were filtered as described in Fig. 1. Total ICYP binding in the absence of competing ligands was 5 fmol. Data are representative of two to three separate experiments performed in triplicate. Variability among experiments averaged 10 to 20%. *A*, ● and ▲ represent data derived from wild type and mutant β-adrenergic receptor, respectively, in the presence of increasing concentrations of (−)-propranolol. ○ and △ represent data derived from wild type and mutant receptor, respectively, in the presence of increasing concentrations of (+)-propranolol. *B*, competition binding to wild type β-adrenergic receptors in the presence of (−)-isoproterenol (○), (−)-epinephrine (□), and (−)-norepinephrine (△). Competition binding to Asn-79 mutant β-adrenergic receptors in the presence of (−)-isoproterenol (●), (−)-epinephrine (■), and (−)-norepinephrine (▲).

shown). In addition, the expression vector also contains the neomycin resistance gene which is expressed from an SV40 early promoter to ensure selection of stably transformed cells when grown on medium containing the neomycin analogue, Geneticin (G-418).

Membranes prepared from cells transfected with expression vector containing the wild type human β-adrenergic receptor and cultured in the presence of 1 μM dexamethasone for 24 h displayed [^{125}I]iodocyanopindolol (ICYP)[1] binding equivalent to 117 ± 7.4 fmol/mg protein (Fig. 1*B*). The calculated K_d for ICYP binding (21 ± 5 pM) is comparable to that reported previously (2). ICYP binding to the mutant Asn-79 receptor displayed a similar receptor density (143 ± 9 fmol/mg protein) and K_d (22 ± 5 pM) as the wild type receptor (Fig. 1*C*).

The interaction of wild type and Asn-79 receptors with adrenergic agonists and antagonists was examined by competition binding of these ligands with ICYP. Wild type and mutant receptors have the same affinity for the (+)- and (−)-isomers of propranolol (Fig. 2*A*); however, the Asn-79 receptor has a significant reduction in affinity for the catecholamines, isoproterenol (40-fold), epinephrine (140-fold), and norepinephrine (240-fold), as compared to the wild type receptor (Fig. 2*B*). The mutant Asn-79 receptor retains stereoselective binding of agonists; the (−)- and (+)-isomers of isoproterenol display approximately the same difference in potency for displacement of ICYP as the wild type receptor (Table I). Similarly, the same rank order of agonist potency is retained in the Asn-79 receptor of (−)-isoproterenol > (−)-epinephrine

TABLE I

Human β_2-adrenergic receptors: affinities for adrenergic agents

The affinity constant (K_d) for the radioligand ICYP was determined directly in equilibrium binding studies. The K_i for competing adrenergic agents was calculated from competition studies using the analysis of Cheng and Prusoff (20). Values represent the mean ± standard error of the mean of the indicated number of experiments (n), with each point performed in triplicate.

	K_d	
	Wild type receptor	Asn-79 receptor
	nM	
(−)-Isoproterenol	17.6 ± 4 (n = 3)	697 ± 26 (n = 3)
(−)-Isoproterenol + Gpp(NH)p	80 ± 7 (n = 2)	650 ± 110 (n = 2)
(+)-Isoproterenol	200 ± 33 (n = 2)	12,167 ± 2,105 (n = 2)
(−)-Epinephrine	56.5 ± 6 (n = 2)	7,800 ± 1,800 (n = 2)
(−)-Norepinephrine	219 ± 27 (n = 2)	52,500 ± 2,500 (n = 2)
(−)-Propranolol	0.22 ± 0.1 (n = 2)	0.22 ± 0.1 (n = 2)
(+)-Propranolol	36.8 ± 3.0 (n = 2)	36.8 ± 0.1 (n = 2)
(±)-Iodocyanopindolol	21 ± 5.0 (n = 2)	22 ± 5.0 (n = 2)

[1] The abbreviations used are: ICYP, [^{125}I]iodocyanopindolol; G_s, stimulatory guanine nucleotide regulatory proteins; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; PGE_1, prostaglandin E_1; PBS, phosphate-buffered saline.

bilize the agonist-receptor interaction even in the absence of the amine counterion (Asp-79); the unsubstituted positively charged amine group of norepinephrine would be unable to participate in a hydrophobic interaction at this site. The observation that antagonist binding is unaffected by this mutation suggests that the binding of agonists and antagonists at this site on the β-adrenergic receptor is fundamentally different.

Strader *et al.* (15) did not observe a similar differential affinity shift for isoproterenol and epinephrine in their study on the substitution of Asp-79 with alanine in the hamster lung β-adrenergic receptor. It is not clear whether the lack of such shift in agonist affinity was due to the aspartate-alanine substitution or to the transient expression system used for assay of the mutant receptor.

The findings that the Asp-79 mutant receptor displays no guanine nucleotide-sensitive agonist binding and affects minimal stimulation of adenylate cyclase imply that the role of this residue in receptor function is not limited to catecholamine binding. Our data suggest that this locus in transmembrane segment II is also involved in an agonist-induced conformational change which facilitates its interaction with G_s. Substitution of Asp-79 with Asn appears to limit the range of possible receptor conformations and thereby prevents the effective transfer of the energy of ligand binding to other effectors. The increases in intracellular cyclic AMP levels observed in the presence of 10–100 μM isoproterenol may reflect a small percentage of Asn-79 mutant receptors randomly assuming a functional conformation in the presence of high agonist concentrations.

All adrenergic and muscarinic cholinergic receptors and mammalian opsins appear to share functional homology in that they transfer the signal of ligand/light binding to effector systems via guanine nucleotide regulatory proteins. Conservation of Asp-79 among members of the gene family implies an important role for this amino acid in receptor function. Our findings from site-directed mutagenesis of Asp-79 suggest that this locus in transmembrane segment II may be involved in the activation mechanism of the β-adrenergic receptor. Mutagenesis studies with other members of this supergene family will be necessary to determine if conservation of this amino acid residue is associated with conservation of receptor mechanisms. The availability of an established cell line expressing Asp-79 mutant β-adrenergic receptors will facilitate further pharmacological and structural studies of this important mutation.

Acknowledgments—We would like to thank Dr. Anthony R. Kerlavage for helpful discussions.

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APPENDIX H

Analysis of the purity of the beta-2 adrenergic peptides I and II provided by Professor L. Visser, University of Pretoria.

D^{79} L V M G L A V V P F G A A H I L M K M^{99} (C - G)

Amino acid	Peptide	n-palmitoyl peptide
Asp (1)	0.85[2]	1.00
Gly (3)	(3.00)[1]	(3.00)[1]
His (1)	0.93[2]	1.03
Ala (3)	2.97	3.16
Pro (1)	1.11	1.44[5]
Val (3)	2.34[2]	2.80[2]
Met (3)	3.61[4]	3.14
Cys (1)	0.63	0.77
Ile (1)	1.03[3]	1.13[2]
Leu (3)	2.84[2]	2.92
Phe (1)	1.05	1.02
Lys (1)	1.02	0.99

1. Ratios calculated i.t.o. Gly = 3.00
2. 72h hydrolysis
3. 48h hydrolysis
4. Contains some residual free methionine used in cleavage of peptide from resin.
5. No resolution between Pro and NH_3

The optical purity of the peptide was determined using enantioselective HPLC. All enantiometric pairs except for D/L Asp could be separated by HPLC using a chiral eluant. The separation is based on the differential distribution between the stationary and mobile phases of D- and L-amino acid complexes, which form with the chiral eluant Cu(II)-N,N' dimethyl-L-Phe.

APPENDIX I

Beta-2 Receptor Peptide ELISA

1. Reagents.

TST Washing Solution:	0.1M NaCl, 0.05M Tris pH 8.0 Tween 20 0.05%
ELISA diluent:	0.5M NaCl 0.01 M Na_2HPO_4 0.5% Triton X-100
Blocking Solution:	1% fetal calf serum in phosphate buffered saline
PBS: (10x stock)	NaCl (80g) KCl (2.0g) $Na_2HPO_4.2H_2O$ (14.4g) KH_2PO_4 (2g)/litre

2. ELISA protocol

1. Coat Titertek plates with 0.5μg peptide/50μl methanol/well overnight. Allow to air dry.
2. Wash the plates 5x with PBS
3. Block with 200μl/well blocking solution for 1 hour
4. Discard blocking solution
5. Add 50μl/well mouse serum/hybridoma harvest fluid diluted in blocking solution, in duplicate and leave for 1 hour at room temperature.
6. Wash 10x with TST.
7. Add 100 μl of a 1/500 goat-α-mouse peroxidase conjugate (Cappel) to each well for 30 minutes.

8. Wash 10x with TST.

9. Add 100μl of ABTS (2,2 Azino bis (3-ethyl benz thiazoline sulphuric acid (Diammonium salt)(Cat. A1888 Sigma) substrate freshly prepared as follows:
0.1M sodium citrate (525mg citric acid monohydrate in 50ml distilled H_2O) adjust pH to 4 ± 0.1 with 3N NaOH. Dilute ABTS (40mM kept stored frozen in aliquots) 1:100 in citrate buffer and add 1/100 volume 30% H_2O_2.

10. Leave for 30 mins and read absorbance at 414 nM.

APPENDIX J

Reagents used for monoclonal antibody production (in section 5.5)

1. Foetal calf serum (FCS) from State Vaccine Institute heated for 30 min at 56°C.
2. RPMI 1640 with glutamine pH 7.0.
3. Human umbilical cord serum (HUCS) pooled heated 56°C for 30 min and filter sterilised.
4. Penicillin 10 000U/ml and streptomycin 10 000 μg/ml in sterile PBS (stored frozen in aliquots) (100x P+S)
5. Polyethelene glycol (PEG) Merck M.W. 4000. 2g PEG/2ml warm RPMI (37°C).
6. Freezing medium 10% dimethyl sulphoxide DMSO and 10% FCS in RPMI (without antibiotics)
7. Aminopterin (Sigma) 100x stock = 1.76 mg/100ml H_2O used at 4 x 10^{-7}M final concentration.
8. HT medium (100x) hypoxanthine $1x10^{-4}$M, thymidine 1.6 x 10^{-5}M glycine $3x10^{-6}$M Na pyruate $1x10^{-3}$M in RPMI.
9. HAT-HUCS medium. 1 ml 100x aminopterin + 1 ml 100x HT + 10 ml HUCS + 1 ml 100x P+S made up to 100ml RPMI.
10. HT-HUCS medium. Same as HAT-HUCS but without aminopterin.
11. Versene solution: EDTA in buffered saline pH 7.4

APPENDIX K

Immunofluorescence of binding of B4A8-PWL to WT-7 Cape Town cells.

Multiwell 10 slides (Titertek) were soaked in detergent (7x) for two hours, and then rinsed once in tap water, three times in doubled distilled water and twice in sterile water. After baking for 3 hrs at 250°C (wrapped in foil) in an oven, they were coated with poly-L-lysine (sterile), 10µl/ml, for two hours and then washed again using tissue culture water before drying in a laminar flow hood.

4 x 10^5 B82 cells (transfected or untransfected cells) were seeded on to the multiwell 10 slides in RP-10 and cultured until almost confluent (2-3 days) and then pulsed overnight with 10^{-6} M dexamethasone (Sigma) followed by immunofluorescence studies on either fixed, or unfixed cells, using a protocol adapted by Dr. A. Khalili-Shirazi.

(i) Protocol for Immunofluorescence of fixed cells

1. Cells were washed 3x with RPMI
2. Cells were fixed with 4% paraformaldehyde in PBS pH 7.4 for 2-3 min at RT.
3. Wash 3x thoroughly with PBS
4. Wash 1x with PBS, 0.1% BSA (wash buffer)
5. Block with 0.1M ethanolamine in PBS 0.1% BSA for 30 min at 4°C.
6. Wash 1x with wash buffer.
7. Treat gently with 0.01% Triton X-100 (1 drop of Triton X-100 in 50ml PBS) at RT for 5 min.
8. Wash 2x with wash buffer
9. Dry around the well and smear silica gel around it.

10. Add B4A8 1/50 dilution (or control antibody) in 5% HS (horse serum) PBS for 60 min at RT
11. Wash 3x with wash buffer
12. Add 1/50 goat anti-mouse whole immunoglobulin FITC (Cappel, USA) for 40 min at RT.
13. Wash 3x with wash buffer.
14. Mount in 30% Glycerol/PBS

(ii) Immunofluorescence of fresh cells

1. Wash slides 3x with RPMI and dry around each well
2. Add 25-50μl of 1st antibody (B4A8 or control 1/50 dil) to each well and leave in a moist chamber for 60 min at 4°C
3. Wash 2x with wash buffer
4. Wash 2x with PBS
5. Fix with 4% paraformaldehyde in PBS pH 7.4 for 2 min at RT
6. Wash 2x with PBS
7. Wash 2x with wash buffer and dry around each well.
8. Block with 5% HS PBS for 30 min at RT
9. Wash 1x with wash buffer
10. Add 1/50 goat anti-mouse whole immunoglobulin FITC (Cappel USA) for 40 min at RT
11. Wash 3x with wash buffer
12. Wet mount (30% glycerol/PBS 0.1% azide)

Slides were viewed under UV light using an Olympus BH-2 fluorescence microscope and photographed at 80x magnification, average speed 40-52.5 seconds exposure using an Olympus C-35AD camera and 400 ASA Fujicolor film.

APPENDIX L

Coupling of Cyanogen Bromide (CNBr) activated Sepharose to sheep anti-mouse immunoglobulin.

500mg of CNBr activated Sepharose 4B (Pharmacia) was suspended in 1mM HCl for a few minutes prior to washing by filtering through Whatman filter paper with 300 ml 1mM HCl. Sepharose 4B was suspended in 2.5 ml coupling buffer (0.84g 0.1M $NaHCO_3$ in 2.9g 0.5M NaCl in 100 ml H_2O) containing 1 mg purified sheep anti-mouse antibody rotated overnight at 4°C. Excess sheep anti-mouse antibody was removed by two centrifugations at 100g for 2 min in coupling buffer. Active groups were blocked by tumbling at room temperature for 2 hours in 1M ethanolamine and then washed in 3 cycles of alternating pH in 0.1M Na acetate 0.5M NaCl pH 4 buffer followed by 0.1M Tris 0.5M NaCl pH 8 and then stored at 4°C in the 0.1M Tris buffer. Prior to use, the beads were washed 3x with PBS.

In order to check that the coupling was successful, 200 μl of the final suspension was incubated with 500μl of a 2mg/ml mouse immunoglobulin preparation kindly provided by Dr. Jill Finlayson (University of Cape Town) for 90 minutes, centrifuged for 2 minutes in a microfuge and after washing 5x with PBS, 20μl of 2x sample buffer was added, the sample was boiled for 2 minutes, Sepharose pelleted again and the supernatant loaded on to 11% PAGE and Western blotted on to Immobilon™. Results of the Western blot in Fig. 92 confirmed that both heavy and light chains of mouse IgG were immunoprecipitated by the CNBr sheep anti-mouse Sepharose beads.

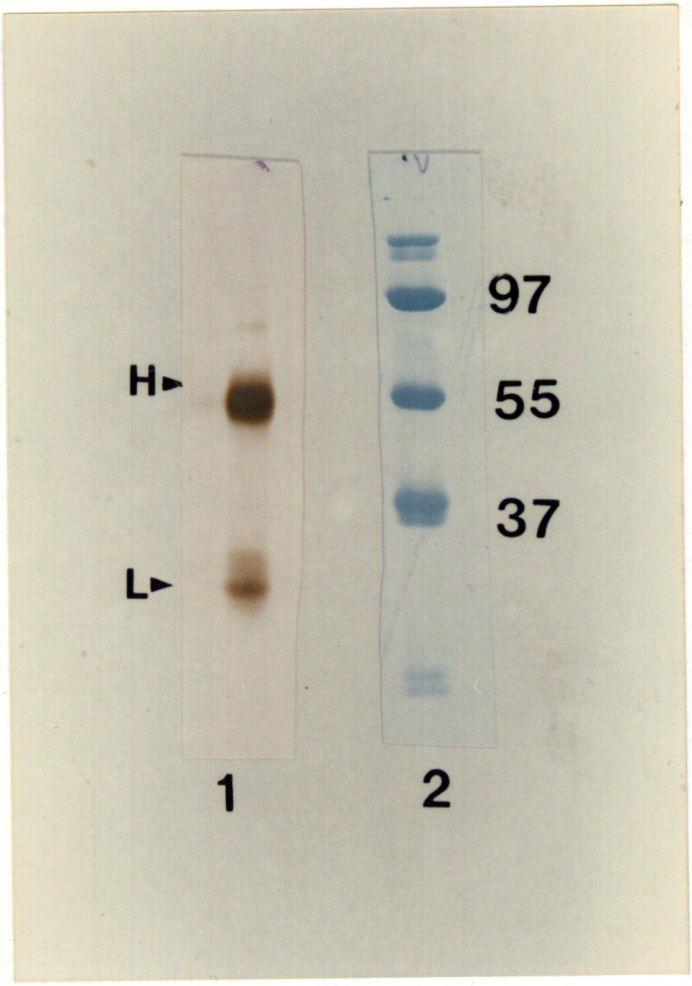

Figure 92. Western blot showing immunoprecipitation of light and heavy chains of mouse IgG by sheep anti mouse Sepharose beads (left lane). Molecular weight markers are shown on the right.

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
National Institutes of Health

Memorandum

Date March 31, 1987

From Chief, Section of Receptor Biochemistry and Molecular Biology, LMCN, IRP, NINCDS

Subject Extention of Appointment of Dr. Paul C. Potter as a Visiting Fellow

To Deputy Director of Intramural Research Program, NINCDS
Through: Director, NINCDS
Scientific Director, IRP, NINCDS
Associate Director for Labs, IRP, NINCDS
Administrative Officer (Labs), IRP, NINCDS
Chief, LNP, IRP, NINCDS

I hereby request that the appointment of Dr. Paul C. Potter as a visiting fellow in the Section of Receptor Biochemistry and Molecular Biology, LMCN be renewed for six months from July 1, 1987 to December 31, 1987.

Dr. Potter joined this laboratory as a visiting fellow in July 1986 and has shown great enthusiasm and dedication to his research. He has a high sense of responsibility and integrates well with other members of this section.

Dr. Potter has initiated and developed techniques for the isolation and quantitation of neuroreceptor RNA which he has applied to a study of beta adrenergic receptor expression in the Wl-38 lung fibroblast cell line at the level of gene transcription. He is also studying Beta adrenergic receptor expresssion in the developing guinea pig fetal lung. He plans to define the structural domains of the Beta adrenergic receptor with which autoantibodies from patients with allergic diseases interact, using cloned beta receptors recently established in an expression system in this laboratory.

In view of Dr. Potter's satisfactory performance and the competence with which his work has been excuted, I request that his appointment as a visiting fellow be extended for a period of six months effective July 1987.

Signed

J. Craig Venter, Ph.D.

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